ANAEROBIC BIODEGRADATION OF SELECTED ORGANIC COMPOUNDS WITH AND WITHOUT INHIBITION OF SULFATE REDUCING BACTERIA

by

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(ABSTRACT)

The primary objective of this study was to investigate the use of hydrogen as a structural substitute or as a reducing equivalent in the anaerobic biodegradation of methanol, methyl tert-butyl ether (MTBE), toluene, phenol, and 2,4-dichlorophenol. In addition, biodegradation rates of these compounds at various initial concentrations with and without inhibition of sulfate reducing bacteria were determined along with anaerobic biodegradation rate constants for each of the compounds studied.

Rates of methanol biodegradation were only slightly altered in molybdate amended microcosms indicating that methanol is a noncompetitive substrate in Blacksburg soil. MTBE biodegradation was slow and followed first order kinetics with respect to initial concentration. Molybdate had no affect on MTBE biodegradation alone, but increased the biodegradation rate in MTBE microcosms which were amended with ethanol. Toluene, phenol, and 2,4-dichlorophenol biodegradation proceeded at two different rate versus initial concentration relationships for lower and upper concentration ranges. Phenol biodegradation followed first order kinetics. The 2,4-dichlorophenol biodegradation rate order varied from 0.78 to 1.75. Monod kinetics were followed by

methanol, toluene, and phenol, but not by MTBE, ethanol amended MTBE, or 2,4-dichlorophenol.

Addition of molybdate to inhibit sulfate reduction increased the degradation rates more for compounds which may require hydrogen in a structural position (2,4-dichlorophenol, MTBE) than those which require hydrogen for proton reduction (methanol). Biodegradation or recalcitrant compounds may be stimulated by the addition of organics (such as ethanol) which produce hydrogen upon biodegradation.

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Chapter 1

Introduction

Groundwater represents more than 96% of all freshwater in the United States, and approximately 50% of the U.S. population uses groundwater as their source of drinking water. Although once thought to be a pristine liquid, recent studies have found concentrations of organic contaminants in groundwater which are far higher than have ever been detected in aboveground water sources (Tangley, 1984). Another fairly recent discovery was the existence of large populations of microorganisms in subsurface soil and underground aquifer systems. "In fact, the total biomass in regions below the root zone in North America is probably much higher than the bacterial biomass in the rivers and lakes of our continent" (Wilson and McNabb, 1983). The abundance of these microorganisms in subsurface environments is fortuitous since "biodegradation is the major mechanism for removal of most organic compounds from soil" (Thornton-Manning et al., 1987).

Many of the aquifers contaminated by xenobiotic compounds are naturally anaerobic or become anaerobic due to the high oxygen demand imposed by the biodegradation of the pollutants. An understanding of the anaerobic biodegradation rates and mechanisms is essential in order to fully evaluate the natural and anthropogenic remediation processes which are available. An integral part of the anaerobic biodegradation process is the competition between sulfate reducing bacteria

and methanogenic bacteria for hydrogen which is used as a reducing equivalent or as a structural substitute (e.g., for dehalogenation) in degradation reactions.

The primary objective of this study was to investigate the use of hydrogen as a structural substitute or as a reducing equivalent in the anaerobic biodegradation of methanol, methyl tert-butyl ether (MTBE), toluene, phenol, and 2,4-dichlorophenol. These compounds are major groundwater pollutants released from leaking underground gasoline storage tanks and pipelines, or discharged as industrial pollutants. In addition, biodegradation rates of these compounds at various initial concentrations with and without inhibition of the sulfate reducing bacteria were determined along with anaerobic biodegradation rate constants for each of the compounds studied.

Chapter 2

Literature Review

2.1 Introduction

The five compounds which were investigated in this study were chosen because of their environmental impact on the nation's groundwater supply and because the biodegradation kinetics of these compounds may be representative of many other similar groundwater pollutants. It has been reported that the initial steps in the biodegradation of 2,4-dichlorophenol may involve the reductive dehalogenation of the ortho and then the para Cl groups (Suflita and Miller, 1985). Therefore, 2,4-dichlorophenol was studied in order to determine the effect of competition between the methanogens and the sulfate reducing bacteria for the hydrogen which would be used in this dehalogenation reaction. Since, after dehalogenation, 2,4-dichlorophenol and phenol have the same biodegradation pathways, the difference between the 2,4-dichlorophenol rate data and the phenol rate data should shed additional light as to how the initial dehalogenation reactions affect the overall biodegradation rates.

Kinetic data has been reported for methanol biodegradation without molybdate (Goldsmith, 1985), and some rate data is available for methanol with molybdate (Morris, 1988). Methanol was chosen for the present study in order to obtain more kinetic data and to compare this data with previously reported data. Toluene and MTBE were chosen because, as yet, no kinetic data is available for these compounds in anaerobic soil systems. Also, MTBE biodegradation kinetics may be comparable to TBA

biodegradation kinetics because both pathways may involve hydrogen substitution for the attached methyl groups.

Following is a summary of the properties of each compound, a description of the production and uses for each compound, and a synopsis of the established EPA ambient water criteria for each applicable compound.

2.2 *Methanol Characteristics*

Methanol, CH_3OH, is a clear liquid with a mild odor at room temperature. Table 2.1 presents some physicochemical data for methanol. Methanol is completely miscible in water. It is volatile, inflammable, and poisonous when taken internally. Methanol is often referred to as wood alcohol because it was obtained commercially from the destructive distillation of wood for over a century (Kirk-Othmer, 1981).

About 7.21 billion pounds of methanol were produced in the United States in 1986 (Anonymous, 1987b). Methanol is prepared commercially from pressurized mixtures of hydrogen, carbon monoxide, and carbon dioxide gases in the presence of metallic heterogeneous catalysts. It is used as a solvent and in the production of formaldehyde, acetic acid, methyl methacrylate, methylamines, methyl halides, dimethyl terephthalate, methyl tert-butyl ether (MTBE), Oxinol (a methanol/tert-butyl alcohol blend used for enhancing gasoline octane), and other chemical intermediates. About half of the methanol presently produced is used to produce formaldehyde, but this will change in the future as greater quantities of acetic acid, MTBE, and Oxinol are produced (Kirk-Othmer, 1981).

The primary sources of methanol contamination of groundwater are leaking underground gasoline storage tanks. Methanol is an oxygenate — a high-octane

Table 2.1
Methanol Physicochemical Data

Physicochemical Property	Property Value
Molecular weight	32.04
Melting point, °C	-97.68
Boiling point at 760 torr, °C	64.70
Vapor pressure at 25°C, torr	127.20
K_{ow}	-0.77
Water solubility at 20°C	miscible
Density at 25°C, g/cm^3	0.7866

Sources: Garrett, 1987; Kirk-Othmer, 1981; Merck, 1983

hydrocarbon which contains oxygen and which is added to gasoline in order to boost or increase the overall octane number. Methanol is miscible in water and is not readily adsorbed to soil. Therefore, like other oxygenates, methanol travels with the groundwater and forms a plume around the dissolved gasoline plume (Garrett, 1987). The problem is further complicated by cosolvency effects because other less-soluble gasoline components (like toluene) are more soluble in the methanol/water mixture than in pure water. Cosolvency effects are most significant in the immediate vicinity of a spill, where the concentration of an oxygenate (methanol) is the highest, and become less important as the oxygenate concentration is diluted (Garrett, 1987). Therefore, the presence of methanol in a gasoline spill can result in a large dissolved methanol plume around the dissolved gasoline/methanol plume and can result in increased concentration of other gasoline components in the groundwater.

2.3 MTBE Characteristics

Methyl tert-butyl ether (MTBE) is a highly volative liquid with the empirical formula $(CH_3)_3C(OCH_3)$. Table 2.2 presents some physicochemical data for MTBE. The proper IUPAC nomenclature for this substance is 2-methoxy-2-methylpropane, but the literature refers to the MTBE designation exclusively.

About 2.24 billion pounds of MTBE were produced in the United States in 1986 (Anonymous, 1987a). It is prepared commercially by reacting methanol with isobutylene (Anderson, 1988). MTBE is primarily used as an octane enhancer for gasoline and is added to gasoline at concentrations up to 15% by volume (Anderson, 1988). MTBE is still new in the western United States but is the most extensively used octane enhancing additive in New England (Garrett, 1987).

Table 2.2
MTBE Physicochemical Data

Physicochemical Property	Property Value
Molecular weight	88.15
Melting point, °C	-109
Boiling point at 760 torr, °C	55.2
Vapor pressure at 25°C, torr	245
K_{ow}	$\simeq$ 1.0-1.5
Water solubility at 25°C, mg/L	43,000
Density at 20°C, g/cm^3	0.7404

Sources: Garrett, 1987; Merck, 1983

MTBE contamination of groundwater creates special problems for the following reasons (Garrett, 1987):

1. MTBE is a more soluble and more rapidly spreading groundwater contaminant than other associated components in gasoline;
2. cosolvency caused by the presence of MTBE in spilled gasoline increases dissolved concentrations of other gasoline components in groundwater in the immediate vicinity of the spill to about an order of magnitude above typical values for spills without MTBE; and
3. oxygenates, in general, are more difficult to remove from contaminated water than the hydrocarbon components of gasoline.

2.4 Toluene Characteristics

Toluene, $C_6H_5CH_3$, is a moncyclic aromatic hydrocarbon with a distinctive odor somewhat milder than that of benzene. Table 2.3 presents some physicochemical data for toluene. Toluene is a colorless liquid at room temperature and is only slightly soluble in water. It is often referred to as part of the BTX (Benzene/Toluene/Xylene) fraction of gasoline.

About 5.82 billion pounds of toluene were produced in the United States in 1986 (Anonymous, 1987a). Toluene is generally produced along with benzene, xylenes, and C_9 aromatics by the catalytic reforming of C_6-C_9 napthas from petroleum. The resulting crude reformate is extracted to yield a mixture of benzene, toluene, xylenes, and C_9 aromatics, which are then separated by fractionation (Kirk-Othmer, 1983). Industrial uses for toluene include the manufacture of benzene derivatives, caprolactum, saccharin, medicines, dyes, perfumes, and trinitrotoluene (TNT). Toluene is also used as a solvent for paints, coatings, gums, resins, rubber and vinyl organosols and as a diluent and thinner in nitrocellulose lacquers (Smith et al., 1987).

Table 2.3
Toluene Physicochemical Data

Physicochemical Property	Property Value
Molecular weight	92.13
Melting point, °C	-95
Boiling point at 760 torr, °C	110.6
Vapor pressure at 25°C, torr	28.7
K_{ow}	2.69
Water solubility at 25°C, mg/L	534.8
Density at 20°C, g/cm^3	0.866

Sources: EPA, 1979; Merck, 1983

Industry uses only a small percentage of the toluene produced each year. Nearly all (90-95%) of the toluene produced annually in the United States is blended directly into gasoline (Kirk-Othmer, 1983). Therefore, the principal sources of toluene contamination in groundwater are leakage from disposed petroleum products in municipal landfills (Wilson et al., 1986b) and leakage from underground gasoline tanks and pipelines (Barker et al., 1987; Raymond et al., 1976; Svoma and Houzim, 1984; Tangley, 1984; Wilson et al., 1986a). Over 8 million gallons of gasoline and crude oil were lost to the environment due to pipeline accidents in 1971 (Raymond et al., 1976), and about 11 million gallons of gasoline are lost each year from underground storage tanks (Tangley, 1984).

Toluene is one of the 129 specific priority chemicals that are considered toxic under the 1977 Amendments to the Clean Water Act (Keith and Telliard, 1979). It is also listed in appendix VIII, part 261, of 40 CFR as a hazardous waste (40 CFR, 1987). The established EPA criterion for toluene states an ambient water limit of 14.3 mg/L as the maximum level allowable to insure protection of public health. For the protection of human health from the toxic properties of toluene ingested through contaminated aquatic organisms alone, the ambient water criterion is determined to be 424 mg/L (EPA, 1980c).

2.5 Phenol Characteristics

Phenol, C_6H_5OH, is a colorless, crystalline solid which has a characteristic taste and odor. Table 2.4 presents some physicochemical data for phenol. Phenol has a relatively high water solubility and a relatively low vapor pressure and octanol-water

Table 2.4
Phenol Physicochemical Data

Physicochemical Property	Property Value
Molecular weight	94.11
Melting point, °C	40.90
Boiling point at 760 torr, °C	181.75
Vapor pressure at 20°C, as a supercooled liquid, torrs	0.53
K_{ow}	1.46
pK_a	10.02
Water solubility at 20°C, mg/L	93,000

Source: EPA, 1979

partition coefficient. Due to the electronegative character of the phenyl group, phenol exhibits weakly acidic properties (EPA, 1980a).

About 3.1 billion pounds of phenol were produced in the United States in 1986 (Anonymous, 1987b). Phenol is prepared commercially by synthetic processes, such as cumene peroxidation, benzene chloration, toluene oxidation, and benzene sulfonation (Babich and Davis, 1981). Phenol or phenolic waste are also produced during the coking of coal, distillation of wood, operation of gas works and oil refineries, manufacture of livestock dips, as a normal constituent of human and animal wastes, and microbiological decomposition of organic matter (EPA, 1980a). Phenol may be produced during the aerobic microbial decomposition of benzene (Gibson et al., 1968), the anaerobic fermentative biodegradation of benzene (Vogel and Grbic-Galic, 1986), and the anaerobic microbial decomposition of chlorinated phenols (Suflita and Miller, 1985).

Commercial products which require phenol include phenolic resins, germicides, pharmaceuticals, fungicides, dyes, herbicides, plastics such as Bakelite®, explosives such as picric acid (2,4,6-trinitrophenol), antiseptic throat lozenges such as Sucrets®, and skin medications such as Campho-phenique® (Smith, 1984). Phenol is also used to produce fertilizers, paints, paint removers, rubber, textiles, and perfumes (Babich and Davis, 1981). Joseph Lister reported on the use of phenol sprays for disinfecting operating rooms in 1867 (EPA, 1980a), and phenol is still commonly used as a disinfectant in dilute solutions for hospital equipment, floors, and walls (Smith, 1984).

Phenol is one of the 129 specific priority chemicals that are considered toxic under the 1977 Amendments to the Clean Water Act (Keith and Telliard, 1979). It is also listed in appendix VIII, part 261, of 40 CFR as a hazardous waste (40 CFR, 1987). The established EPA criterion for phenol states an ambient water limit of 3.5 mg/L as

the maximum level allowable to insure protection of public health. A maximum stream concentration of 0.3 mg/L is established for controlling undesirable taste and odor qualities of ambient water (EPA, 1980a).

2.6 2,4-Dichlorophenol Characteristics

2,4-Dichlorophenol (2,4-DCP) is a colorless, crystalline solid with the empirical formula $C_6H_3Cl_2OH$. Table 2.5 presents some physicochemical data for 2,4-DCP. 2,4-DCP has a relatively low water solubility, vapor pressure, and octanol-water partition coefficient. It behaves as a weak acid and is highly soluble in alkaline solutions, since it readily forms the corresponding alkaline salt (EPA, 1980b).

About 86 million pounds of 2,4-DCP are produced each year in the United States (Smith et al., 1987). 2,4-DCP is prepared commercially by direct chlorination of phenol. As an intermediate in the chemical industry, 2,4-DCP is principally used as the feedstock for the production of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4-D derivatives (germicides, soil sterilants, etc.) and certain methyl compounds used in mothproofing, antiseptics, and seed disinfectants. It is also used to produce miticides and pentachlorophenol, a wood preservative (EPA, 1980b).

2,4-DCP is one of the 129 specific chemicals that are considered toxic under the 1977 Amendments to the Clean Water Act (Keith and Telliard, 1979). It is also listed in appendix VIII, part 261, of 40 CFR as a hazardous waste (40 CFR, 1987). The established EPA criterion for 2,4-DCP states an ambient water limit of 3.09 mg/L as the maximum allowable to insure protection of public health. A maximum stream concentration of 0.3 μg/L is established for controlling undesirable taste and odor qualities of ambient water (EPA, 1980b).

Table 2.5
2,4-Dichlorophenol Physicochemical Data

Physicochemical Property	Property Value
Molecular weight	163.0
Melting point, °C	45
Boiling point at 760 torr, °C	210
Vapor pressure at 20°C, calculated, torr	0.12
K_{ow}	2.75
pK_a	7.85
Water solubility at 20°C, mg/L	4,500

Source: EPA, 1979

2.7 *Microbial Biodegradation of Selected Organic Compounds — Introduction*

Groundwater contamination by man-made organic chemicals is a serious problem. Many of the aquifers contaminated by xenobiotic compounds are naturally anaerobic or become anaerobic due to the high oxygen demand imposed by the biodegradation of the pollutants. Therefore, an understanding of the anaerobic biodegradation rates and mechanisms is essential in order to fully evaluate the natural and anthropogenic remediation processes which are available.

Anaerobic biodegradation of methanol, phenol, and 2,4-DCP has been shown in many studies. Until recently, toluene was thought to be recalcitrant to biological degradation without the presence of molecular oxygen. However, recent field evidence and microcosm studies have demonstrated anaerobic biodegradation of toluene. MTBE is a relatively new groundwater pollutant and, as such, no biodegradation studies have been reported for MTBE alone. The following is a review of the relevant biodegradation studies of the compounds mentioned above.

2.8 *Methanol Biodegradation*

Methanol biodegradation in anaerobic environments has been extensively studied. Methanol readily degraded in subsurface soil samples from Virginia, Pennsylvania, and New York so that initial concentrations up to 1000 mg/L were reduced to nonmeasurable levels in one year or less (Goldsmith, 1985; Novak et al., 1985). The presence of tert-butyl alcohol (TBA) or TBA and benzene, toluene, and xylene (BTX) had no effect on methanol degradation in these samples.

White et al. (1986) found that previous contamination of an aquifer by gasoline containing TBA had no observed effect on methanol biodegradation. Similarly, Goldsmith et al. (1985) found that methanol biodegradation kinetics at low methanol concentrations can be approximated by a first-order equation and that the kinetics are similar for uncontaminated aquifers and for aquifers previously contaminated with gasoline containing TBA. However, at higher methanol concentrations, biodegradation kinetics for the uncontaminated and contaminated sites were found to differ.

Whether sulfate reducing bacteria (SRB) compete with methanogens for methanol is contradictory in the literature. Braun and Stolp (1985) could not isolate pure strains of SRB with methanol as the sole source of carbon and energy. Experiments with sulfate reducing and methanogenic aquifer materials indicated that acetate, H_2, and formate were competitive substrate materials, whereas methanol and trimethylamine were converted to methanol (Beeman and Suflita, 1987). No SRB capable of growing on methanol or trimethylamine were detected in either type of material. Lovley and Klug (1983b) found that methanogens were primarily responsible for the metabolism of methanol, monomethylamine, and trimethylamine in the sediments of a eutrophic lake and that sulfate reducers were poor competitors for methanol and methylamines in sulfate-amended sediments. Similarly, Oremland et al. (1982) found that methanol and trimethylamine were important substrates for methanogenic bacteria in anoxic salt marsh sediments, but that these compounds did not stimulate sulfate reduction. Also, Oremland and Polcin (1982) found that SRB do not compete with methanogens for methanol, trimethylamine, or methionine in estuarine sediments taken from intertidal mud flats.

Although the literature cited above seems to indicate that SRB do not compete effectively with methanogens for methanol, there is also evidence to the contrary. SRB are known to utilize methanol as an electron donor (Postgate, 1979). King (1984) reported that SRB outcompeted MB for methanol at low (μM) concentrations in marine sediments, but that the reverse was true at higher concentrations (> 1 mM). Results from experiments with molybdate and 2-bromoethanesulfonic acid (BESA) suggested that methanol was primarily oxidized through sulfate reduction in sediments from an intertidal zone in Maine (King et al., 1983). Also, results from experiments with molybdate in slurries of salt marsh sediments suggested that competition was occurring between methanogens and SRB for methanol (Banat et al., 1983).

2.9 MTBE Biodegradation

No study of MTBE biodegradation has been reported in the literature. Fujiwara and others found that the presence of MTBE had little effect on the biodegradability of blended gasoline, but they did not discuss the biodegradability of MTBE alone (Fujiwara et al., 1984 cited in Garrett, 1987). Alexander (1981) reported a general biodegradation cleavage reaction for ethers in soil. This reaction is shown below:

$$ROCH_2R' \rightarrow ROH \qquad (2.1)$$

Several cases of groundwater contamination with MTBE have been reported. A township in north-central New Jersey was forced to install an aeration treatment system and a granular activated carbon system due to the sudden presence of MTBE, diisopropyl ether (DIPE), and other gasoline contaminants in the township's groundwater supply (McKinnon and Dykson, 1984). MTBE is almost the only

octane-enhancing additive used in Maine and is reported to be a significant groundwater pollutant at gasoline spill sites in that state (Garrett, 1987; Garrett et al., 1987). Garrett (1987) reported on a gasoline spill from a farmer's tank in southwestern Maine. Water from some wells within 750 feet of the spill contained as much as 690 ppb MTBE, but no detectable gasoline, two years after the spill occurred.

2.10 Toluene Biodegradation

The aerobic biodegradation of toluene by soil microorganisms is well documented (Gibson et al., 1968; Kappeler and Wuhrmann, 1978; Swindoll et al., 1988a; Swindoll et al., 1988b; Tabak et al., 1964). Most of the degradation studies have been done with bacteria fixed to soil, but free-living groundwater bacteria have also been reported to degrade toluene and other aromatic hydrocarbons and heterocyclic compounds (Arvin et al., 1988). Aerobic in situ decontamination of hydrocarbon polluted aquifers (including toluene) has been demonstrated. Raymond (1974) received a patent on a process designed to remove hydrocarbon contaminants from groundwater by stimulating the indigenous microbial population with nutrients and oxygen (aeration). The process was applied at a pipeline leak site in Ambler, Pennsylvania (Raymond et al., 1976). No gasoline could be detected in the groundwater ten months after the period of nutrient addition with continuous aeration — up to 1000 barrels of high octane gasoline were degraded.

Batterman (1986) reported a study of decontamination of an oil-polluted aquifer by microbial degradation. Ammonia (1 mg/L), orthophosphate (0.3 mg/L), and nitrate (up to 500 mg/L) were injected into the aquifer to stimulate the growth of bacteria. During the 2 years of clean-up operations a total of 100 tons of nitrate were used,

leading to the biodegradation of 30 tons of hydrocarbons. Toluene was completely removed from the aquifer within eight months. Hilberts et al. (1986) report one other in situ biorestoration project where nutrients and aeration were used to stimulate biodegradation of gasoline components in an aquifer. Approximately two tons of hydrocarbons were degraded within one year.

Until recently aromatic hydrocarbons were thought to be recalcitrant to biological degradation without the presence of molecular oxygen (Young, 1984) or oxygen-containing substituent groups (Evans, 1977). Schink (1985a) reported that benzene and toluene moderately inhibited methanogenesis in sediment slurries. Schink (1985b) also reported that there was no indication of benzene, toluene, or xylene (BTX) biodegradation in anaerobic enrichment cultures after 14 weeks of incubation. Similarly, Barker et al. (1987) did not observe BTX biodegradation in anaerobic microcosms after 60 days of incubation.

However, field evidence from an aviation gasoline spill site suggests that natural aerobic and anaerobic in situ biorestoration of groundwaters contaminated with petroleum products can occur (Wilson et al., 1986a). Anaerobic degradation of toluene and other aromatic hydrocarbons has been demonstrated in microcosms using aquifer material from beneath an aviation spill site (Wilson et al., 1986a), from beneath a landfill (Wilson et al., 1986b), and from previously uncontaminated aquifers (Wilson et al., 1983). Grbic-Galic and Vogel (1987) demonstrated concomitant degradation of ^{14}C-labeled toluene and benzene and the production of $^{14}CO_2$ under methanogenic conditions. Toluene and *m*-xylene were rapidly mineralized in an anaerobic aquifer column operated under continuous-flow conditions with nitrate as an electron acceptor (Kuhn et al., 1988). Also, Vogel and Grbic-Galic (1986) showed the incorporation of

oxygen from water into toluene and benzene during anaerobic fermentation of these substances.

2.11 Phenol Biodegradation

Phenol biodegradation under aerobic conditions has been studied extensively. Phenol has been shown to biodegrade, and kinetic constants have been determined in continuous activated sludge systems (Baird et al., 1974; Beltrame et al., 1980; Beltrame et al., 1982; Beltrame et al., 1984; Yang and Humphrey, 1975) and batch activated sludge systems or chemostats (Beltrame et al., 1979; Sokol and Howell, 1981; Yang and Humphrey, 1975). Aerobic biodegradation has been shown in estuarine water systems (Banerjee et al., 1984; Hwang et al., 1986; Lewis et al., 1988; Shimp and Pfaender, 1987; Visser et al., 1977) and soil systems (Portier and Fujisaki, 1986; Scott et al., 1982; Scow et al., 1986; Swindoll et al., 1988a; Swindoll et al., 1988b; Tabak et al., 1964; Varga and Neujahr, 1970). Since the present study involves anaerobic degradation of phenol in subsurface soil, a more intensive review of the anaerobic degradation literature is given below.

Groundwater contamination from two wood treatment facilities provided opportunities for in situ studies of anaerobic degradation of phenol. Troutman et al. (1984) and Goerlitz et al. (1985) reported on phenolic contamination of a sand-and-gravel aquifer from a surface impoundment of wood treatment wastes in Pensacola, Florida. They found a rapid, nearly exponential, decline in total phenol concentration in shallow groundwater from 1,000 μg/L, at a distance 650 feet downgradient from the source, to 10 μg/L, 800 feet downgradient from the source. Elevated levels of dissolved methane in the groundwater and elevated counts of

methanogenic bacteria relative to populations of sulfate reducing bacteria at sites downgradient from the surface impoundment suggested that an anaerobic methanogenic ecosystem was in part responsible for degradation of phenols in the groundwater.

Ehrlich et al. (1982) studied the degradation of phenolic contaminants from a coal-tar distillation and wood-treating plant in St. Louis Park, Minnesota. Under the ambient conditions of the groundwater, over 95% of the phenolic compounds were removed within 1000 m of the contamination source. This was attributed to anaerobic bacteria due to the following observations:

1. methane was present in the contaminated portions of the aquifer but not elsewhere;
2. methane-producing bacteria were present in areas where methane was found but not elsewhere; and
3. methane was produced from aqueous extracts of fluid from a well inoculated with microbes from the contaminated zone and incubated under anaerobic conditions.

Several microcosm studies have been performed to study phenol degradation under anaerobic conditions. Dobbins et al. (1987) measured mineralization of phenol at 12 concentrations ranging from 5 to 10,000 μg/kg dry soil in 21 separate soil samples from 4 contrasting soil profiles in north-central Alabama. The kinetic terms V_{max} and K′ were determined using nonlinear regression: V_{max} values ranged from 0.018 to 22.4 ng phenol/kg dry soil/s, and k′ values ranged from 20 to 2355 μg phenol/kg dry soil. The effects of temperature and nitrogen, phosphorus, and carbon enrichments on phenol mineralization were determined in two of the contrasting soil profiles mentioned above (Thornton-Manning et al., 1987). Temperature affected mineralization differently as a function of soil type, as did the nitrogen, phosphorus, and carbon enrichments. Mineralization generally exhibited first-order kinetics in these soils.

Smith and Novak (1987) used microcosms to study the subsurface biodegradation rates of phenol and four chlorinated phenols in soil samples from Virginia and Pennsylvania. They found that biodegradation rates increased as initial concentration increased and that all biodegradation rates appeared to follow first-order kinetics with regard to the initial compound concentrations. Biodegradation for the five compounds studied followed the order phenol = 2-chlorophenol > 2,4,6-trichlorophenol > 2,4-dichlorophenol, while pentachlorophenol degraded similarly to 2,4,6-trichlorophenol and 2,4-dichlorophenol.

Young and Rivera (1985) used high performance liquid chromatography to determine the aromatic metabolites of phenol, phloroglucinol, hydroquinone, and *p*-cresol under methanogenic conditions. They found that anaerobic metabolism of the substituted phenols appears to conform to a general scheme in which the ring substituents are removed to yield phenol as an intermediate, ring saturation takes place, followed by fission, yielding organic acid intermediates which serve as methane precursors. A similar mechanism, termed reductive dehalogenation, was reported by Gibson and Suflita (1986). Suflita and Miller (1985) found that microorganisms in an actively methanogenic aquifer were able to metabolize mono-, di-, and tri-chlorophenols by replacing the halogen substituents with hydrogen atoms. However, no dehalogenation occurred in a nonmethanogenic (possibly sulfate-reducing) site.

2.12 2,4-Dichlorophenol Biodegradation

2,4-DCP has been shown to enter the environment by several means. It may enter the environment as a contaminant with the chemical waste from the manufacture of 2,4-D (Montgomery et al., 1972). It is produced and enters the environment when water or wastewater containing phenol is chlorinated (EPA, 1979; EPA, 1980a; EPA, 1980b; Smith et al., 1987). Also, 2,4-DCP may be produced as a biodegradation product of 1,2,4-trichlorobenzene (Marinucci and Bartha, 1979) or 2,4,-D (Gibson and Suflita, 1986; Smith, 1985).

2,4-DCP biodegradation under aerobic conditions has been studied extensively. It has been shown to biodegrade and kinetic constants have been determined in continuous activated sludge systems (Beltrame et al., 1980; Beltrame et al., 1982). Aerobic biodegradation has been shown in estuarine water systems (Baker et al., 1980; Banerjee et al., 1984; Hwang et al., 1986) and soil systems (Baker et al., 1980; Ross, 1980). Goldstein et al. (1985) found that certain aerobic microorganisms (*Pseudomonas* srains) which are able to degrade a pollutant (2,4-DCP) in culture sometimes may fail to function when inoculated into natural environments, possibly because the pollutant concentration in nature may be too low to support growth or because the organisms may be susceptible to toxins or predators in the environment or they may use other organic compounds in preference to the pollutant. Also, inoculated organisms may be unable to move through soil to sites containing the pollutant.

Boyd and Shelton (1984) investigated the biodegradation of 2,4-DCP in fresh and acclimated anaerobic sewage sludge. In fresh sludge, they found that reductive dechlorination of the Cl ortho to phenolic OH occurred first. Thus, 2,4-DCP degraded to 4-chlorophenol. Sludges acclimated to 2-chlorophenol or 4-chlorophenol were also

able to degrade 2,4-DCP. When ^{14}C-labeled 2,4-DCP was added to these acclimated sludges, the mineralization products were found to be 40% $^{14}CH_4$ and 60% $^{14}CO_2$. In each case, over 90% of the total added radioactivity was recovered as $^{14}CH_4$ or $^{14}CO_2$.

Gibson and Suflita (1986) found that 4-chlorophenol was formed when 2,4-DCP was biodegraded by microorganisms from a methanogenic aquifer, an anaerobic pond sediment, or an anaerobic sewage sludge digester. Microorganism samples from a sulfate-reducing site of a groundwater aquifer could degrade benzoate and phenol but were not able to degrade 2,4-DCP. Similarly, Suflita and Miller (1985) found that the orthochlorine was removed from 2,4-DCP and replaced by a hydrogen atom in samples from an actively methanogenic aquifer. No transformation of 2,4-DCP was noted in a nonmethanogenic (possibly sulfate-reducing) aquifer site. As previously mentioned in Section 2.11, Smith and Novak (1987) used microcosms to study the anaerobic subsurface biodegradation rates of phenol and four chlorinated phenols. They found that biodegradation rates increased as initial concentration increased, that all biodegradation rates appear to follow first-order kinetics with regard to the initial compound concentrations, and that 2,4-DCP had the lowest biodegradation rate of the compounds tested.

2.13 Soil Characteristics and Microbial Enumeration

The soil used in the present study was obtained from a relatively pristine location on the Virginia Polytechnic Institute and State University dairy farm at a depth of approximately 15 feet. This soil was not analyzed to determine its major anion and cation constituents, but data from a similar soil is available. Morris (1988) analyzed soil obtained from an adjacent location on the same dairy farm also at a depth of 15 feet.

The results are presented in Table 2.6. The soil used in the present study and the soil used by Morris both consisted mainly of tightly packed unsaturated clay.

Morris enumerated bacterial populations in the dairy farm soil. Spread plate techniques were chosen over the acridine-orange (A-O) direct count method because the soil consisted mainly of clay. It has been found that clay particles are difficult to distinguish from bacteria in a fluorescent sample (White, 1986), so direct counting was not used. Using spread plate techniques, $3.4 \pm 0.2 \times 10^5$ colony forming units (cfu) per gram of soil were measured using yeast extract, while $3.8 \pm 2.0 \times 10^5$ cfu per gram of soil were counted with soil extract as the growth media.

Hickman (1988) reports results of a modified spread-plate technique and a substrate specific MPN test for Blacksburg, Virginia soil. In the modified spread-plate technique, soils were saturated with water and incubated for different periods of time before plating to produce saturated viable counts. Viable counts became relatively constant after two to five days of saturation, and saturating the soils increased viable counts by roughly an order of magnitude. According to Hickman, saturating the soils enhances nutrient availability and relieves moisture stress, which may lead to growth of active microorganisms, germination and growth of inactive forms, and/or increased recovery of viable but nonculturable cells.

In the substrate specific MPN test, methanol or phenol was added to the MPN tubes as the sole carbon source in an attempt to quantify the bacterial population responsible for degrading that chemical in soil. According to this technique, there are 10^5-10^6 methanol degraders and 10^2-10^5 phenol degraders per gram of soil in Blacksburg subsurface environments. A thorough analysis of the relationship between bacterial

Table 2.6
Blacksburg Soil Constituents (15 foot depth)

Constituent	Value
Ca^{+2}, mg/l	15.7
Mg^{+2}, mg/l	1.02
Na^{+}, mg/l	0.33
K^{+}, mg/l	2.22
Fe, mg/l	< 0.03
Al, mg/l	< 0.10
SO_4^{-2}, mg/l	6.70
NO_3^{-}, mg/l	--
NO_2^{-}, mg/l	--
pH	4.2

Source: Morris, 1988

counts, bacterial counting techniques, soil depth, and biodegradation rates may be found in Hickman (1988).

2.14 Microbial Competition for Hydrogen

Respiration is the oxidation of an energy source coupled with reduction of an external electron acceptor. In aerobic respiration, molecular oxygen is the terminal electron acceptor. Aerobic respiration stops and anaerobic respiration may begin when molecular oxygen is depleted in subsurface environments. Two of the major terminal electron acceptors used in anaerobic respiration are sulfate (used by sulfate reducing bacteria) and carbon dioxide (used by methanogenic bacteria).

Competition between sulfate reducing bacteria (SRB) and methanogenic bacteria (MB) for hydrogen is well known. The half-saturation constant, K_s, for hydrogen metabolism by methanogens has been reported to be 6 μm, whereas it is only 1 μm for sulfate reduction (Kristjansson et al., 1982). Thus, sulfate reduction to hydrogen sulfide is energetically favored over methane production, and this explains the apparent inhibition of methanogenesis when the hydrogen concentration is limiting (Speece, 1983). SRB have been shown to outcompete MB for hydrogen in estuarine sediments (Oremland and Polcin, 1982; Winfrey and Ward, 1983), in salt marsh sediments (Abram and Nedwell, 1978b; Banat et al., 1981; Banat et al., 1983; Nedwell and Banat, 1981), in eutrophic lake sediments (Lovley et al., 1982; Smith and Klug, 1981), in oligotrophic lake sediments (Lovley and Klug, 1983a), and in anaerobic sewage isolates (Kristjansson et al., 1982) and other isolated bacterial cultures (Abram and Nedwell, 1978a).

Since SRB normally outcompete MB in hydrogen limited environments, one method to study the role of SRB and MB in degrading groundwater pollutants is to

preferentially provide hydrogen to one population by selectively inhibiting the other population. MB have been inhibited by the addition of 2-bromoethanesulfonic acid (BESA) to microcosm samples (Alperin and Reeburgh, 1985; King et al., 1983; Morris, 1988; Oremland et al., 1982). SRB have been inhibited in sediment samples by incubation of the samples in mineral salts medium which lacked sulfate ions (Oremland and Polcin, 1982) and by inhibition of acetate utilization by the addition of fluorolactate (Abram and Nedwell, 1978b; Alperin and Reeburgh, 1985; Banat et al., 1981; Winfrey and Ward, 1983). Most notably, however, SRB are inhibited by addition of oxyanions of elements from group VI of the periodic table, i.e., analogs of SO_4^{2-}. These oxyanions destroy adenosine 5' -triphosphate (ATP) in cells of sulfate-reducing bacteria, with an approximate order of $CrO_4^{2-} > MoO_4^{2-} = WO_4^{2-} > SeO_4^{2-}$ (Taylor and Oremland, 1979). Molybdate (added as Na_2MnO_4) is the most frequently cited of the oxyanions for inhibition of SRB in microcosm studies (Alperin and Reeburgh, 1985; Banat et al., 1981; Banat et al., 1983; Lovley and Klug, 1983b; Lovley et al., 1982; Morris, 1988; Nedwell and Banat, 1981; Smith and Klug, 1981; Sörensen et al., 1981; Winfrey and Ward, 1983).

Morris (1988) investigated the effect of molybdate and BESA on biodegradation of methanol, tert-butyl alcohol (TBA), phenol, and 2,4-dichlorophenol in subsurface soil samples from Blacksburg, Virginia. Addition of BESA decreased biodegradation by up to 70% of nonamended rates whereas molybdate consistently increased biodegradation rates. The most dramatic increases due to molybdate addition were for TBA (1390%) and 2,4-dichlorophenol (307%). This was attributed to the use of hydrogen for structural substitution (e.g., for dehalogenation) rather than simply the use of hydrogen for reducing equivalents.

2.15 *Graphical Methods*

The microcosms used in this study represent individual batch systems. Since plots of the degradation patterns of batch systems can have several shapes, it is important to use a standard approach for calculating substrate utilization rates from these plots and to recognize the inaccuracies inherent in using this approach for comparisons between soils. Morris (1988) used the method shown in Figure 2.1 to calculate substrate utilization rates. These rates were normalized by dividing each rate (mg L^{-1} day^{-1}) by the amount of soil in each microcosm (mg L^{-1} day^{-1} g^{-1}). This method was also used in the present study.

Lawrence and McCarty proposed the following relationship between the substrate utilization rate and the biomass concentration in biological wastewater treatment systems.

$$\left(\frac{d[S]}{dt}\right)_u\left(\frac{1}{x}\right) = \frac{k[S]}{K_s + [S]} = q \qquad (2.2)$$

where:

$\left(\frac{d[S]}{dt}\right)_u$ = substrate utilization rate, mg L^{-1} day^{-1}

k = maximum substrate utilization rate, day^{-1}

x = biomass concentration, mg L^{-1}

q = specific substrate utilization rate, day^{-1}

[S] = growth limiting substrate concentration, mg L^{-1}

K_s = saturation constant, mg L^{-1}

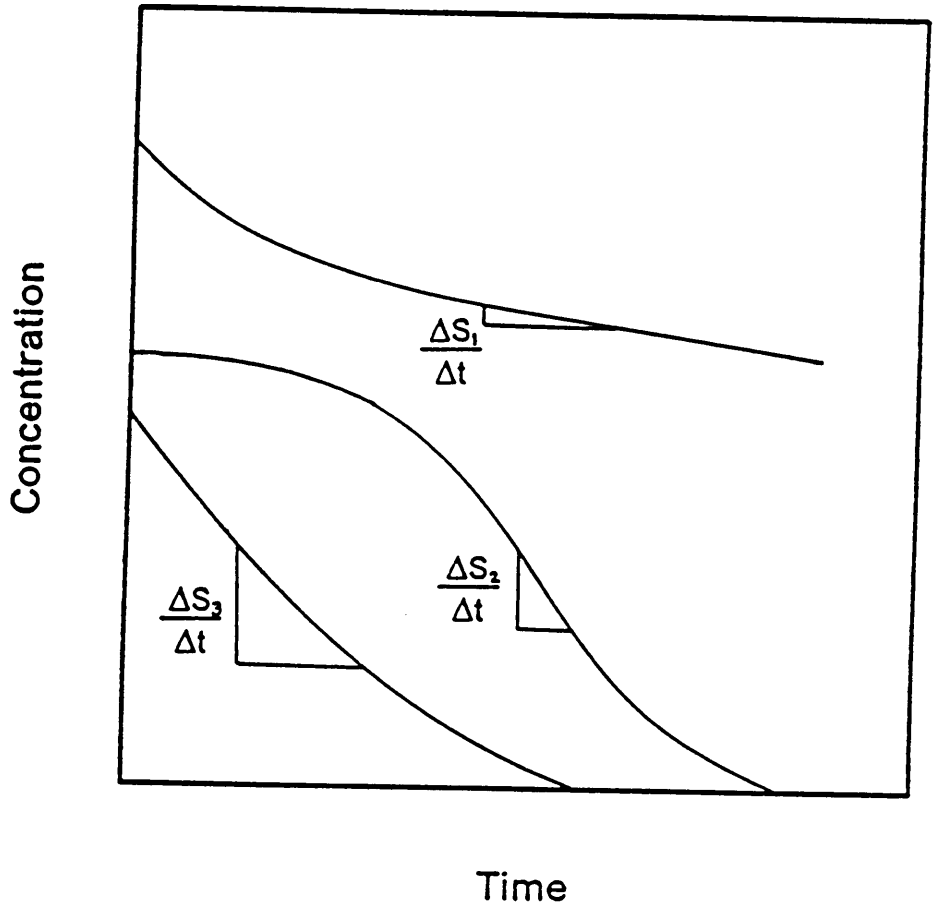

Figure 2.1. Method for determining biodegradation rates
Source: Morris, 1988

In this equation, substrate utilization is a function of organism and substrate concentration over the entire range of substrate concentrations (Morris, 1988). Figure 2.2 shows a graphical representation of this relationship.

The value of q may be calculated for steady state systems as (Goldsmith, 1985):

$$q = \frac{\Delta S/\Delta t}{x} \tag{2.3}$$

where:

ΔS = substrate utilized, mg L^{-1}

Δt = time, days

In this study, an x value specific for each substrate being studied could not be determined, so values of q are taken as $\Delta S/\Delta t$ and normalized by dividing each rate by the amount of soil in each microcosm.

Equation 2.2 is the equation for a rectangular hyperbola, and the constant terms k and K_s are not easily determined from this plot (Figure 2.2). The kinetic constants k and K_s can be determined by using the Lineweaver-Burke double reciprocal modification of equation 2.2.

$$\frac{1}{q} = \left(\frac{K_s}{k}\right)\left(\frac{1}{[S]}\right) + \frac{1}{k} \tag{2.4}$$

A plot of $\frac{1}{q}$ vs. $\frac{1}{[S]}$ yields a straight line with a slope equal to $\frac{K_s}{k}$ and a y-intercept equal to $\frac{1}{k}$. This equation can be accurately used when the [S] values are all within a narrow range (e.g., 1-10 mg/L). When the [S] values are spread over a large range (e.g., 1-100 mg/L), the data points for the higher [S] values cluster near the origin, and the data points for the lower [S] values have the most influence upon the slope.

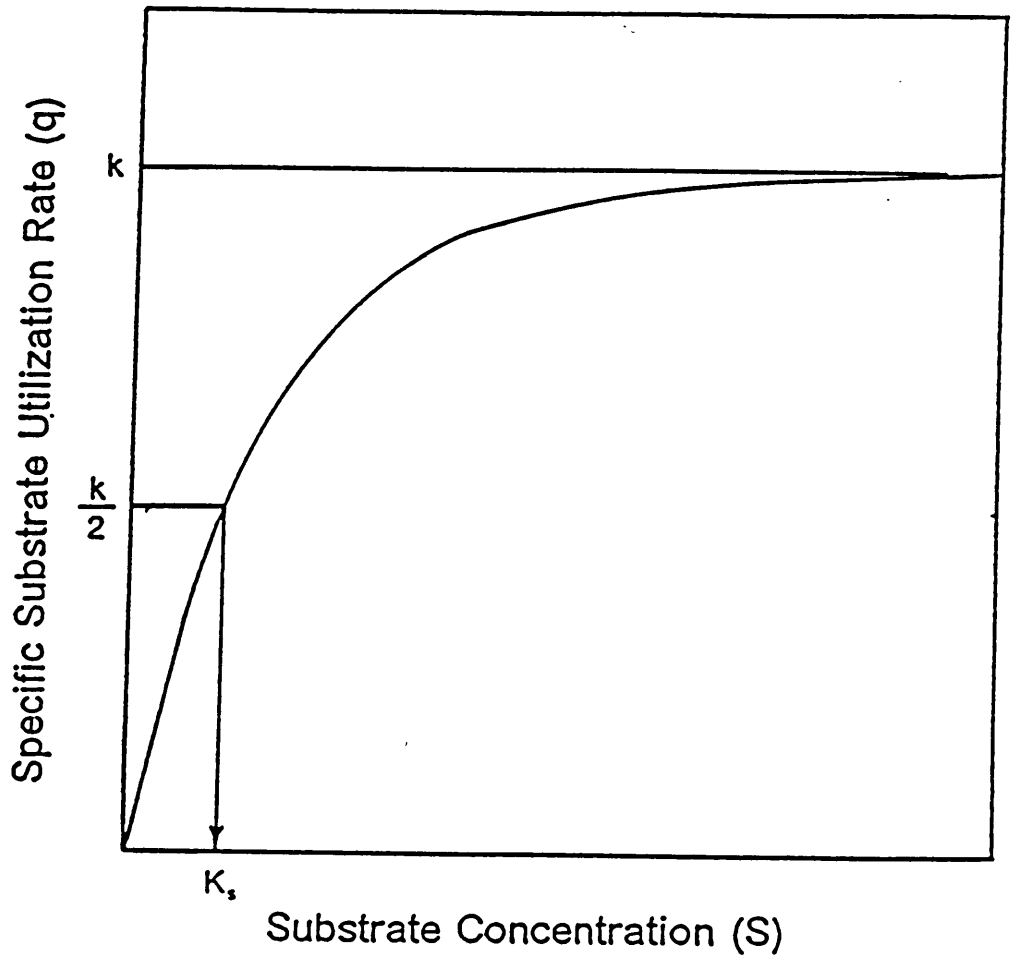

Figure 2.2. Graphical representation of the Monod equation

Another equation which may be used to determine k and K_s is obtained by multiplying both sides of the Lineweaver-Burke equation by [S]:

$$\frac{[S]}{q} = \frac{[S]}{k} + \frac{K_s}{k} \tag{2.5}$$

Thus, a plot of $\frac{[S]}{q}$ vs. [S] yields a straight line with a slope of $\frac{1}{k}$ and a y-intercept of $\frac{K_s}{k}$. This equation was originally suggested by Hanes (Grady and Lim, 1980), and it has the advantage of spreading out the data points obtained from high [S] values so that a more accurate slope may be determined. Equations 2.4 and 2.5 were used to determine the kinetic constants k and K_s in this study.

The rate of a reaction is given by the substrate concentration raised to the nth power as shown below:

$$q = [S]^n \tag{2.6}$$

Logarithmically, this relation can be expressed as follows:

$$\log(q) = n \log([S]) \tag{2.7}$$

Therefore, a log-log plot of degradation rate versus initial concentration will have a slope equal to the reaction order, n.

Chapter 3

Materials and Methods

3.1 Experimental Approach

Table 3.1 shows the experimental matrix which was used to accomplish the objectives outlined in the Introduction above. The values shown in Table 3.1 represent the desired initial concentrations of duplicate (sometimes quadruplet) microcosms. Numerous measurements of the residual organic concentration were performed for each microcosm in order to establish biodegradation rates for each compound. In general, the lowest initial concentration limits were determined by the sensitivity of the packed-column gas chromatography equipment. The intermediate and upper limit concentrations were selected so as to obtain sufficient data to make the desired plots (log utilization rate vs. log initial concentration) and to define the rate constants k and K_s over a wide range of initial concentrations.

The initial results with MTBE were not as expected because the molybdate amended microcosms gave essentially the same biodegradation rates as the nonamended microcosms over the entire concentration range. One reason for this phenomenon may have been that there was insufficient hydrogen in these microcosms for the previously proposed hydrogen competition between SRB and MB to take place. Therefore, additional experiments were performed in which ethanol and acetic acid were added to selected MTBE microcosms. The ethanol and acetic acid were to act as hydrogen sources for the SRB and the MB so that the proposed competition could take place.

Table 3.1
Experimental Matrix of Initial Concentrations for the Compounds Examined in This Study

	Initial Concentration (*mg/L*)										
Compound	**8000**	**3000**	**800**	**600**	**400**	**300**	**80-100**	**20-40**	**8-10**	**3**	**1**
Methanol	X	X	X		X		X		X		
MTBE				X		X	X		X		X
Toluene						X	X	X	X		
Phenol			X		X		X	X	X	X	X
2,4-Dichlorophenol					X		X	X	X	X	X

Note: Each initial concentration was attained in duplicate or quadruplet microcosms. This matrix was also used for microcosms amended with molybdate (1.0 mM as MoO_4^{2-}).

3.2 General Methods

The following general methods were used during the course of this study. All glassware was acid washed in 10% HCl for 24 hours and autoclaved for 30 minutes at 121°C and 15 psi pressure prior to each use. The distilled water used to prepare the organic solutions for biodegradation studies was autoclaved for 30 minutes at 121°C and 15 psi pressure. All utensils used in handling the subsurface material were flame sterilized or autoclaved for 30 minutes at 121°C and 15 psi pressure.

3.3 Site Location and Sample Collection

Subsurface samples were obtained from a previously uncontaminated site on the Virginia Polytechnic Institute and State University dairy farm in Blacksburg, Virginia. The first and second samples were obtained from recently excavated 9 foot deep holes. A shovel was used to excavate 2 additional feet of soil, and a hand auger was used to burrow 4 more feet into the ground. Samples were then taken from a 15 foot depth using the hand auger. A third set of samples were obtained from a site adjacent to the 9 foot deep holes using a hand auger with a 15 foot extension. For all three samples, a sterilized spatula was used to scrape the soil from the hand auger into a sterilized metal pan. The samples were then transferred to sterilized mason jars fitted with teflon caps, and were transported to the laboratory in cooled ice chests. All samples were stored in an incubator at 10°C.

3.4 *Microcosms*

Microcosms were constructed of 13x100 mm screw-capped test tubes sealed with solid caps (for toluene and MTBE) or with 12 mm teflon-coated septum caps (for methanol, MTBE, phenol, and 2,4-dichlorophenol). Each microcosm contained 5-7 grams of soil. The test compounds were diluted with distilled water to the desired concentrations and introduced into the microcosms as the only carbon source. Each microcosm was thoroughly mixed once with a vortex mixer. Microcosms of a particular organic concentration were prepared in duplicate. All microcosms were stored in the dark at a constant temperature of 20°C. Sodium molybdate (1.0 mM as MoO_4^{2-}) was added to some tubes to inhibit sulfate reduction.

To assess the impact of nonbiological processes such as adsorption, volatilization, and chemical degradation on the loss of substrate, control microcosms were prepared for MTBE and toluene. These contained soil which was autoclaved for 30 minutes each day for 7 consecutive days at 121°C and 15 psi pressure. During the first five days, the soil was autoclaved and stored in an aluminum covered metal pan. The soil was transferred to test tubes and autoclaved two more times to kill any bacteria which may have been introduced while loading the microcosms.

After the initial MTBE experiments were completed, ethanol and acetic acid were added to some of the MTBE microcosms to act as a possible source of hydrogen. The additions were made by transferring appropriate amounts of ethanol and acetic acid from stock solutions (10,000 mg/L) into the microcosms using a 10 μL syringe. The microcosms were thoroughly mixed with a vortex mixer after the additions were completed.

3.5 *Analytical Methods*

Because of the difference in volatilities of the test compounds, different procedures were used to obtain samples for analysis. Previous studies have shown that methanol (Novak et al., 1985), phenol, and 2,4-dichlorophenol (Smith, 1984) can be continuously sampled through teflon septa without significant losses due to volatilization. Therefore, these compounds were sampled by puncturing the septa several times with a 10 μL syringe over the life of the experiment. To prevent the introduction of bacteria during the sampling process, the syringe needle was heat sterilized and the septa caps were cleaned with isopropyl alcohol.

Preliminary tests with MTBE in the present study showed measurable volatilization losses of MTBE through punctured septa. In order to decrease volatilization, two sampling procedures were attempted. One set of tubes were sealed using solid screw caps with a teflon septum pushed up into each cap. A tube was sampled by removing the screw cap in a nitrogen-purged container, quickly drawing approximately 2 μL of the aqueous supernatant into a 10 μL syringe and replacing the screw cap on the tube. Another set of tubes were sealed with 12 mm teflon-coated septa. A tube was sampled by puncturing the septum with a 10 μL syringe needle. After each tube was sampled, the septum was replaced with a new septum in a nitrogen-purged container. All replacement septa were autoclaved for 30 minutes at 121°C and 15 psi pressure prior to use. To prevent the introduction of bacteria during the sampling process, the syringe needle was heat sterilized and the septa caps were cleaned with isopropyl alcohol.

Previous experiments with toluene (Farmer, 1989; Farmer et al., 1988) showed that toluene rapidly escaped from tubes with punctured septa. Therefore, the toluene

tubes were sealed using solid caps with a teflon septum pushed up into each cap, and the sampling procedure outlined above was followed.

The concentration of each compound was measured by gas-liquid chromatography using a flame ionization detector in a Model 5880A Hewlett Packard gas chromatograph. A 6′ x 1/8″ stainless steel column packed with 0.2% Carbowax 1500 on 80/100 mesh Carbopack C was used to identify methanol, ethanol, MTBE, and toluene. Phenol and 2,4-dichlorophenol were measured using a 2m x 2mm glass column packed with 1% SP 1240 DP on 100/120 Supelcoport. The carrier gas was nitrogen, and the sample size was 2 μL. Each compound was measured isothermally at temperatures selected to provide sufficient detection and separation from the aqueous phase. The following oven temperatures were used: methanol, 80°C; MTBE, 120°C; toluene, 150°C; phenol, 130°C; 2,4-dichlorophenol, 140°C. An oven temperature of 90°C was used for the MTBE/ethanol/acetic acid tests in order to separate the MTBE, water, and ethanol peaks. For all compounds, the injector port temperature was 150°C, and the detector temperature was 225°C.

Chapter 4

Results

4.1 Introduction

In this section, representative data for each organic compound are presented to illustrate the biodegradation pattern over time of each of the compounds studied. The biodegradation rate data for each compound will also be graphically presented.

4.2 Methanol Results

Figure 4.1 shows the biodegradation data for methanol in microcosms at an initial concentration of approximately 80 mg/L. Methanol biodegradation appeared to begin without a lag phase at this concentration and at most of the other concentrations. A constant and steady decrease in methanol concentration with time was noted in all of the microcosms. The biodegradation rate was slightly higher for the molybdate amended microcosm data shown in Figure 4.1. In general, however, molybdate had little effect on the biodegradation rate of methanol. This is shown more clearly in Figure 4.2.

Figure 4.2 presents the biodegradation rate data from all of the methanol dosed microcosms. Biodegradation rates from duplicate microcosms were averaged and plotted as single points in this figure. In general, the biodegradation rate increased as the methanol initial concentration increased. Molybdate only slightly increased the

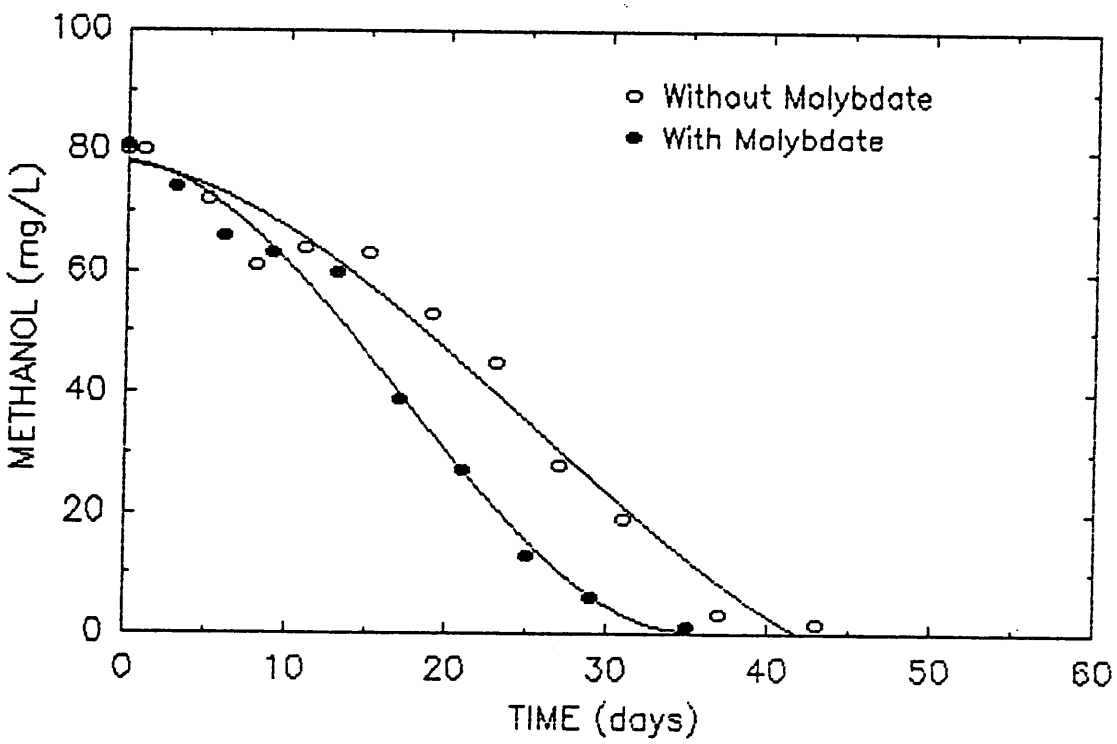

Figure 4.1. Methanol biodegradation in soil microcosms with and without molybdate

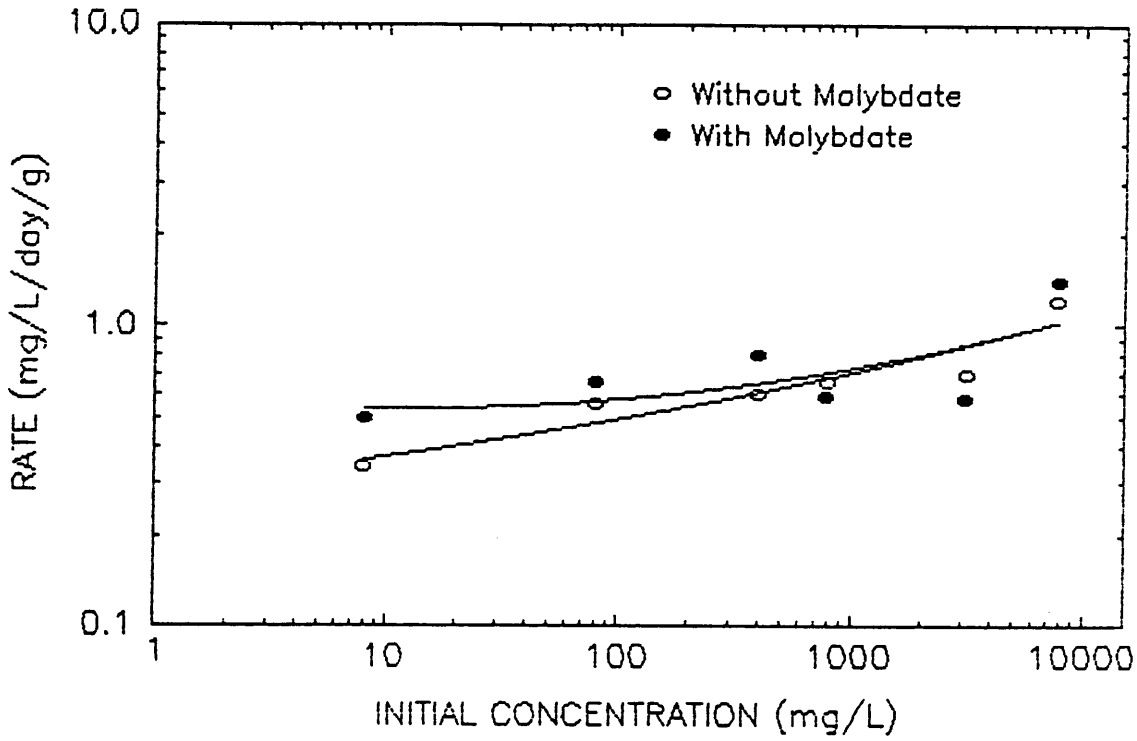

Figure 4.2. Methanol utilization rate in soil microcosms with and without molybdate

biodegradation rates at methanol concentrations below 400 mg/L and had little or no effect on the biodegradation rates at higher concentrations.

4.3 MTBE Results

Figure 4.3 shows example biodegradation data for MTBE with and without molybdate along with data from a control microcosm. MTBE biodegraded very slowly at this concentration (0.046 mg L^{-1} day^{-1} g^{-1}), and the recalcitrant behavior indicated in Figure 4.3 was found to be true at all concentrations. The biodegradation rate was higher (0.075 mg L^{-1} day^{-1} g^{-1}) for the molybdate amended microcosm data shown in Figure 4.3, but, in general, molybdate had little effect on the biodegradation rate of MTBE.

Figures 4.4 and 4.5 show volatilization loss rate data from the septum cap and solid cap control microcosms, respectively. Volatilization losses occurred when the septum caps were changed and when the solid caps were removed to take samples. As shown, the volatilization loss rates increased linearly with the MTBE initial concentration. MTBE biodegradation rates were determined by subtracting the volatilization loss rate obtained from Figure 4.4 or 4.5 from the total MTBE loss rate for each microcosm and then normalizing this rate by dividing it by the number of grams of soil in the microcosm.

Figure 4.6 presents the biodegradation rate data from all of the MTBE microcosms. Biodegradation rates from duplicate microcosms were averaged and plotted as single points in this figure. Figure 4.6 shows that the MTBE biodegradation rate increased as the initial MTBE concentration increased. Molybdate had little effect on the biodegradation rates over the entire experimental concentration range.

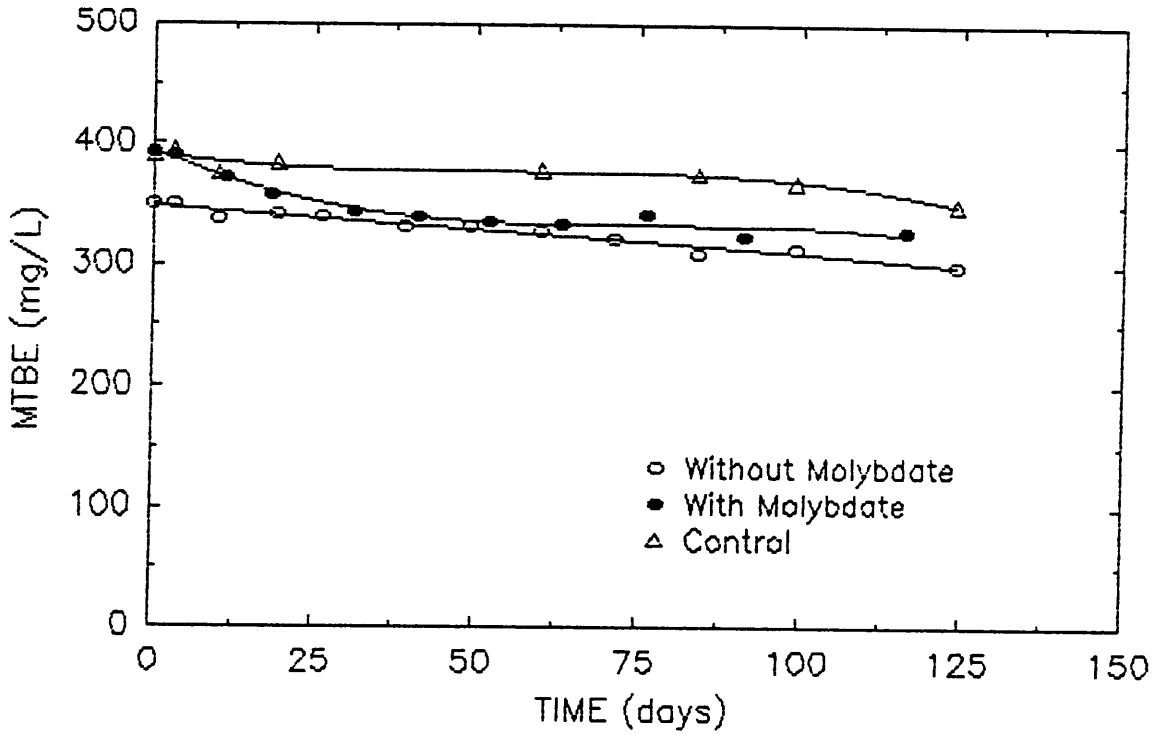

Figure 4.3. MTBE biodegradation in soil microcosms with and without molybdate

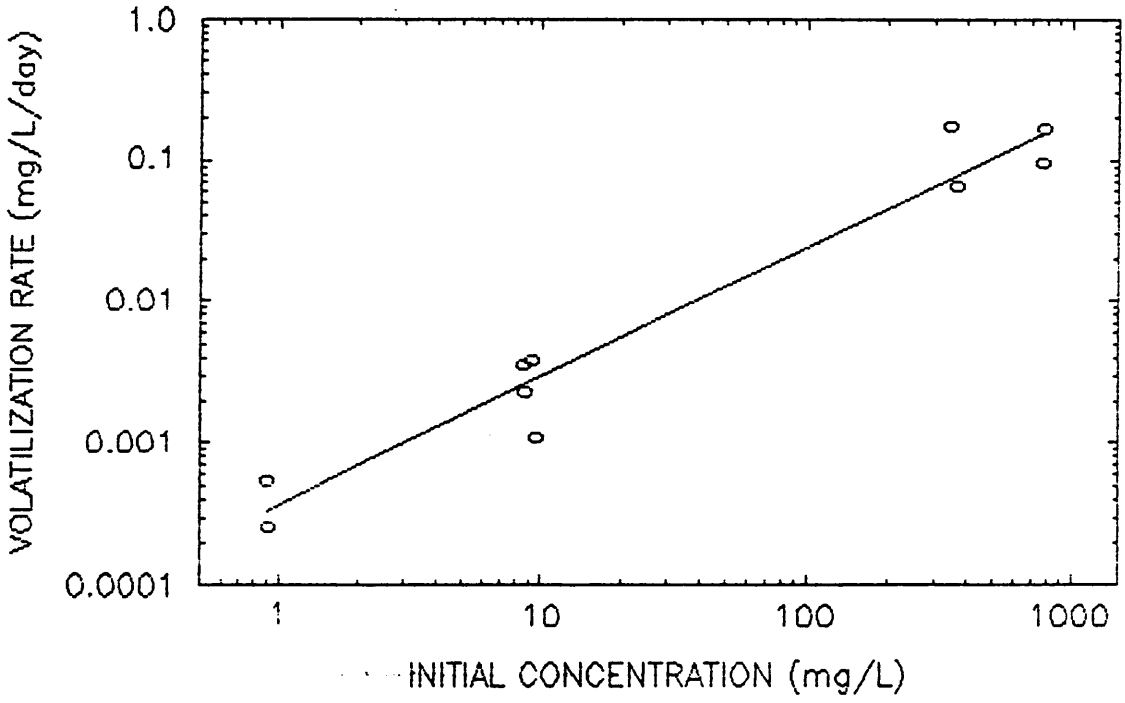

Figure 4.4. Volatilization loss rate from septum capped MTBE microcosms

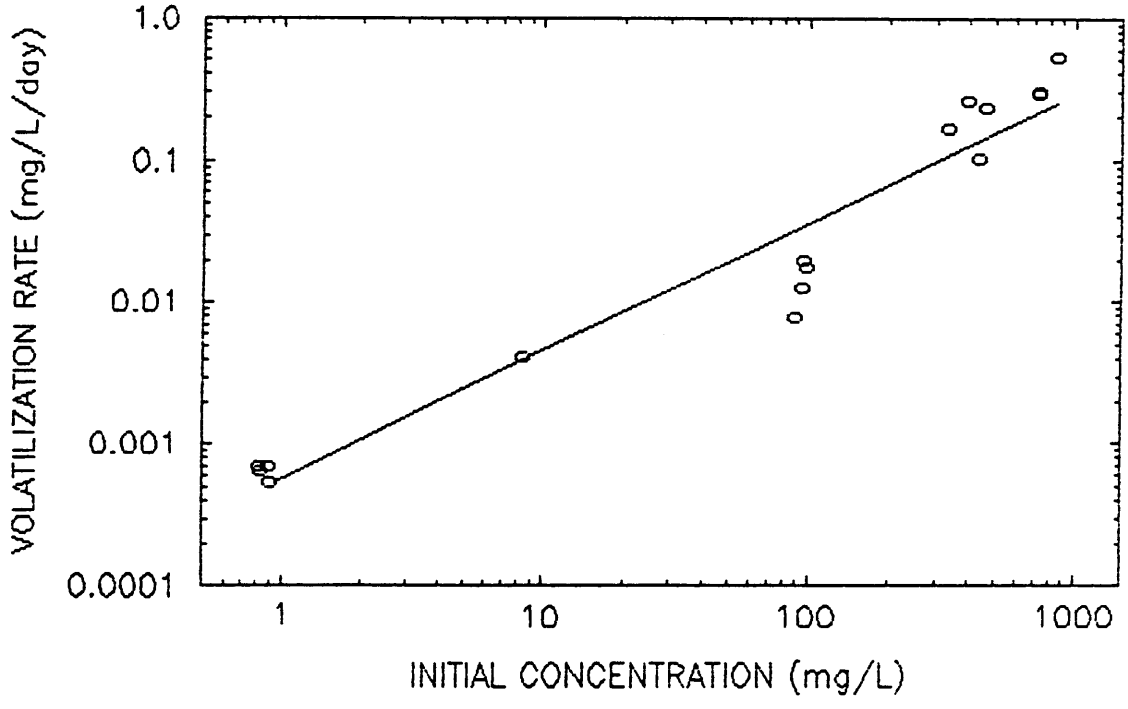

Figure 4.5. Volatilization loss rate from solid capped MTBE microcosms

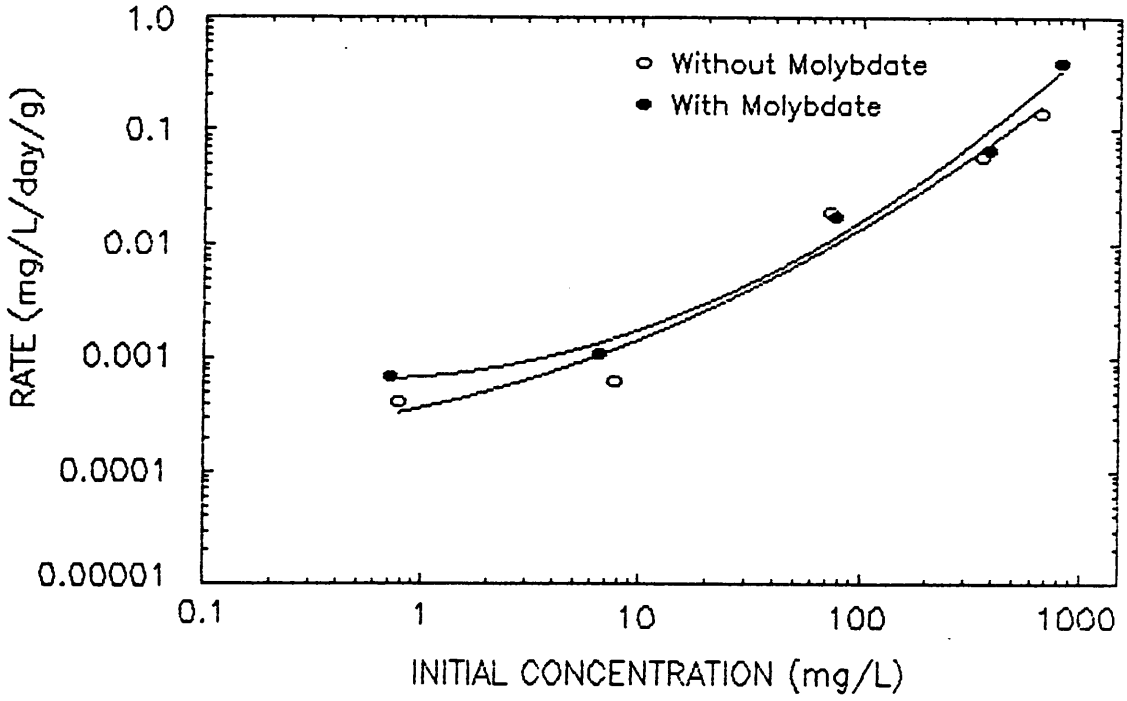

Figure 4.6. MTBE utilization rate in soil microcosms with and without molybdate

4.4 Organic Amended MTBE Degradation

After the initial MTBE experiments were completed, several microcosms containing MTBE were dosed with 30 mg/L ethanol and 30 mg/L acetic acid. These microcosms were dosed a second time with 30 mg/L ethanol after the initial dose of ethanol was biodegraded. Figure 4.7 shows the data from two of the organic amended microcosms. The first dose of ethanol degraded within 16 days in the molybdate amended microcosms, and the MTBE biodegradation rate increased upon addition of ethanol. In the microcosms without molybdate, complete ethanol biodegradation took more than 18 days, and the MTBE biodegradation rate remained approximately the same upon ethanol addition.

Figure 4.8 presents the biodegradation rate data from all of the organic amended MTBE microcosms. Volatilization losses were subtracted prior to plotting these data in the same manner as the nonorganic amended MTBE data. Biodegradation rates from duplicate microcosms were averaged and plotted as single points in this figure. As shown in Figure 4.8, the biodegradation rate increased as the MTBE initial concentration increased. Molybdate increased the biodegradation rate for all of the experimental concentrations, but the two curves in Figure 4.8 converge as the MTBE concentration increases. This indicates that molybdate has less effect on the MTBE biodegradation rate at higher concentrations.

All of the points in Figure 4.8 represent biodegradation over the same time period except for the lowest molybdate data point (5.8 mg/L). Unlike all of the other microcosms, biodegradation of ethanol and MTBE in the two microcosms containing 5.8 mg/L MTBE slowed after 7 days. Therefore, the rate shown in Figure 4.8 is the MTBE biodegradation rate over the first seven days of incubation. MTBE control

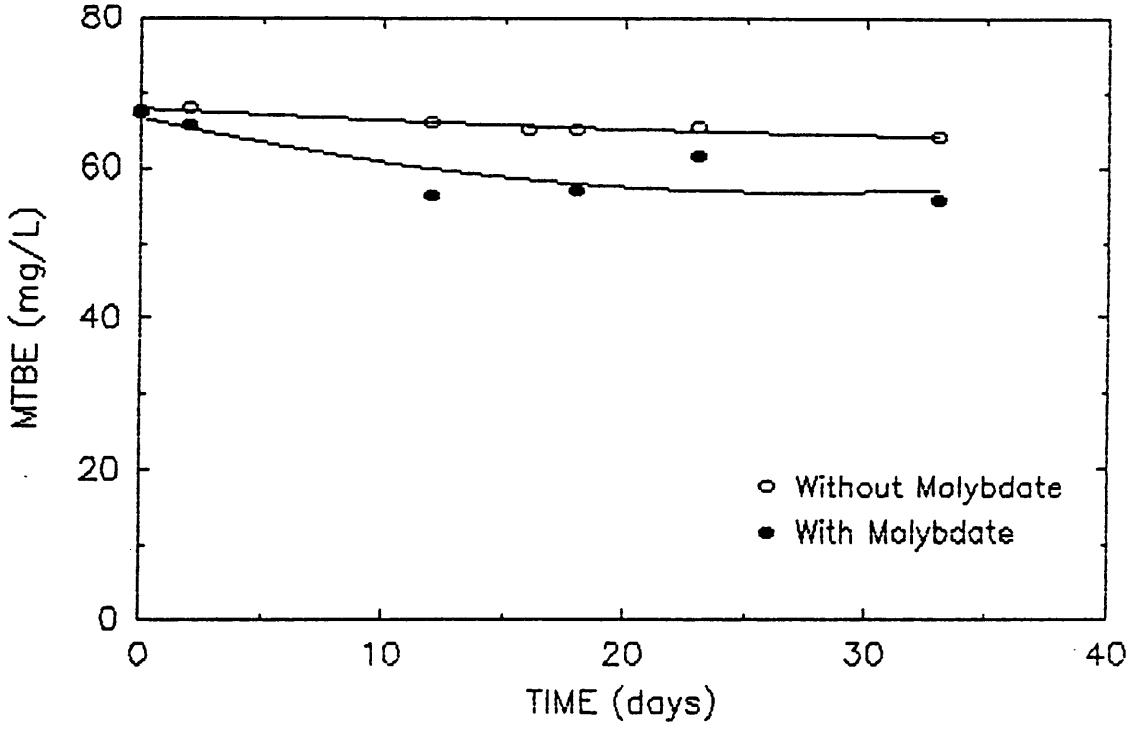

Figure 4.7. Organic amended MTBE biodegradation in soil microcosms with and without molybdate

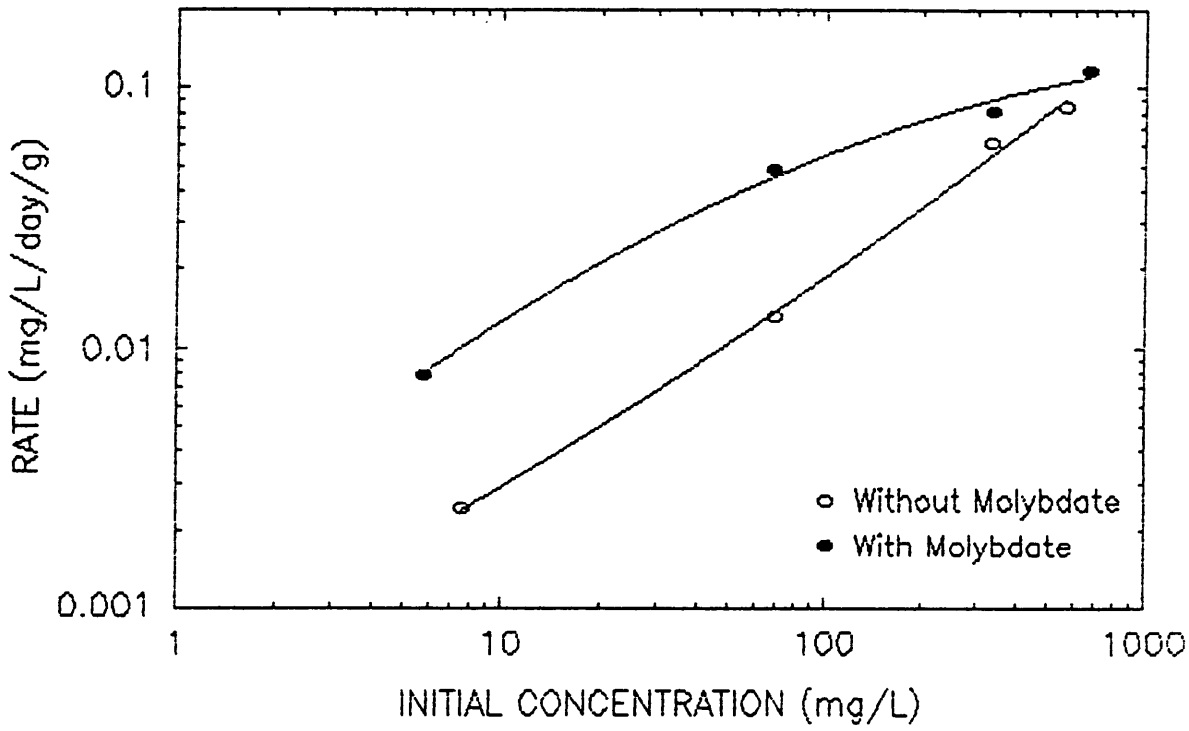

Figure 4.8. Organic amended MTBE utilization rate in soil microcosms with and without molybdate

microcosms were also dosed with ethanol and acetic acid, but the controls became contaminated as evidenced by the biodegradation of ethanol. Therefore, the controls used were from earlier studies without ethanol amendment.

4.5 Toluene Results

Figure 4.9 shows the biodegradation data for toluene at 40 mg/L with and without molybdate along with data from a control microcosm. Toluene biodegradation appeared to start without a lag phase at this concentration and at most of the other concentrations, but some of the initial toluene loss was likely due to adsorption (Farmer et al., 1988). The biodegradation rate was 22% higher for the molybdate amended microcosm data shown in Figure 4.9. The effect of molybdate at other concentrations is considered below in relation to Figure 4.11.

Figure 4.10 shows the volatilization loss rate data from the toluene control microcosms. Volatilization losses occurred when the caps were removed to obtain samples. As shown in Figure 4.10, the volatilization rates increased linearly with the initial concentration of toluene. Toluene biodegradation rates were determined by the same method as outlined above for MTBE. Figure 4.11 presents the biodegradation rate data from all of the toluene microcosms. Biodegradation rates from duplicate microcosms were averaged and plotted as single points in this figure. The data in this figure are shown as representing two different relationships between degradation rate and initial concentration. The toluene biodegradation rates appear to increase with increasing concentration up to an initial concentration of 20-40 mg/L. Beyond a 40 mg/L initial toluene concentration, the biodegradation rates appear to decrease before increasing again. Molybdate increased the biodegradation rate 67% in microcosms with

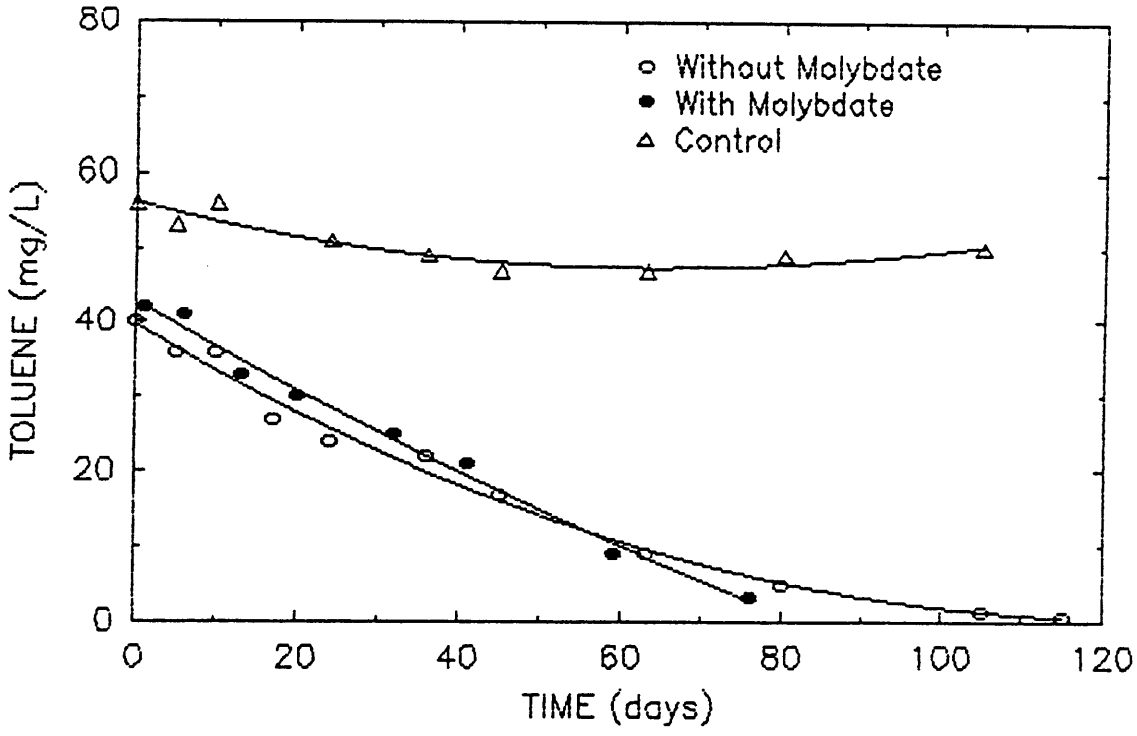

Figure 4.9. Toluene biodegradation in soil microcosms with and without molybdate

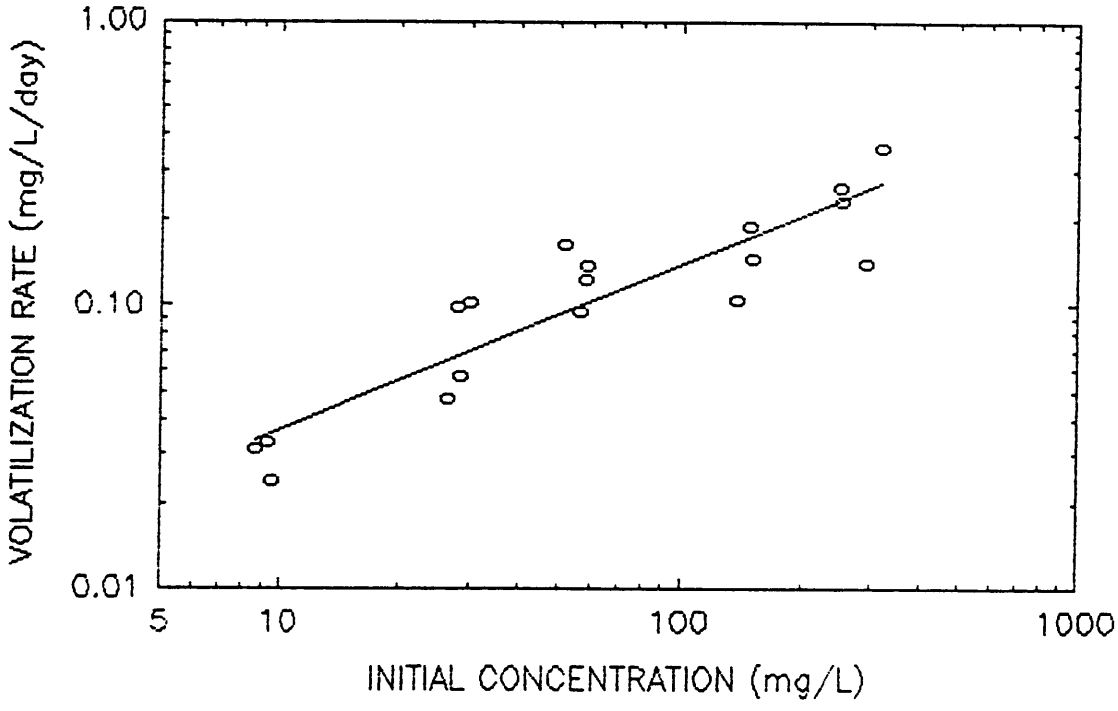

Figure 4.10. Volatilization loss rate from toluene microcosms

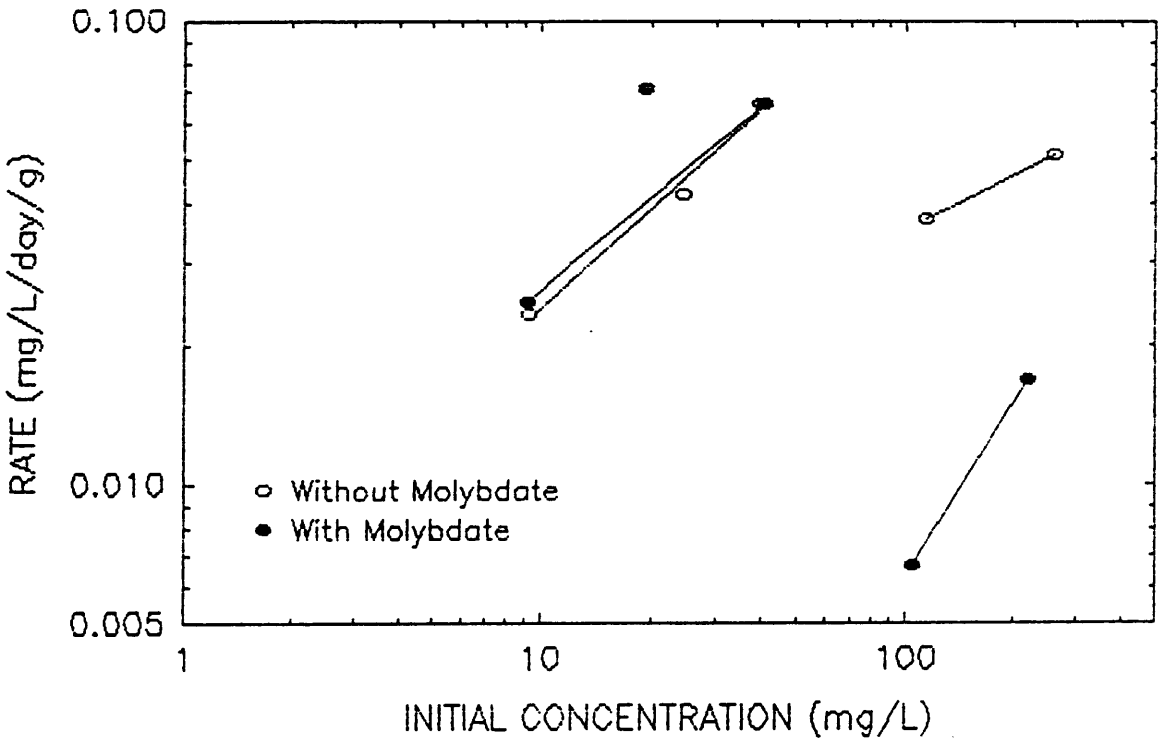

Figure 4.11. Toluene utilization rate in soil microcosms with and without molybdate

an initial toluene concentration of 20 mg/L, but had no effect on biodegradation rates at toluene concentrations of 9 mg/L and 40 mg/L. Lower biodegradation rates were determined for the molybdate amended microcosms than for the nonmolybdate amended microcosms at toluene concentrations greater than 40 mg/L. The data in Figure 4.11 appear to represent two different rate vs. concentration relationships, possibly due to the degradation effects of two different microbial populations at low and high toluene concentrations.

4.6 Phenol Results

Figure 4.12 shows the complete biodegradation of phenol starting from an initial concentration of 90 mg/L. Phenol biodegradation appeared to begin without a lag phase at this concentration, and biodegradation began with little or no lag phase at most other concentrations as well. A constant decrease in phenol concentration was noted in these microcosms (0.18 mg L^{-1} day^{-1} g^{-1} without molybdate, 0.23 mg L^{-1} day^{-1} g^{-1} with molybdate) and in the microcosms with phenol concentrations below 90 mg/L. However, as shown in Figure 4.13, the microcosms with a phenol concentration of 850 mg/L exhibited two separate biodegradation rates -- a higher rate up to 40 days of incubation (1.46 mg L^{-1} day^{-1} g^{-1} without molybdate, 3.40 mg L^{-1} day^{-1} g^{-1} with molybdate) and a lower rate after 40 days of incubation (0.81 mg L^{-1} day^{-1} g^{-1} without molybdate, 0.91 mg L^{-1} day^{-1} g^{-1} with molybdate). A decrease in rate with incubation time also occurred at the 430-450 mg/L phenol concentration and may have been due to nutrient limitations in the soil. Therefore, the higher rates were assumed to be more applicable to groundwater aquifers receiving nutrient recharge, and these rates are plotted in Figure 4.14.

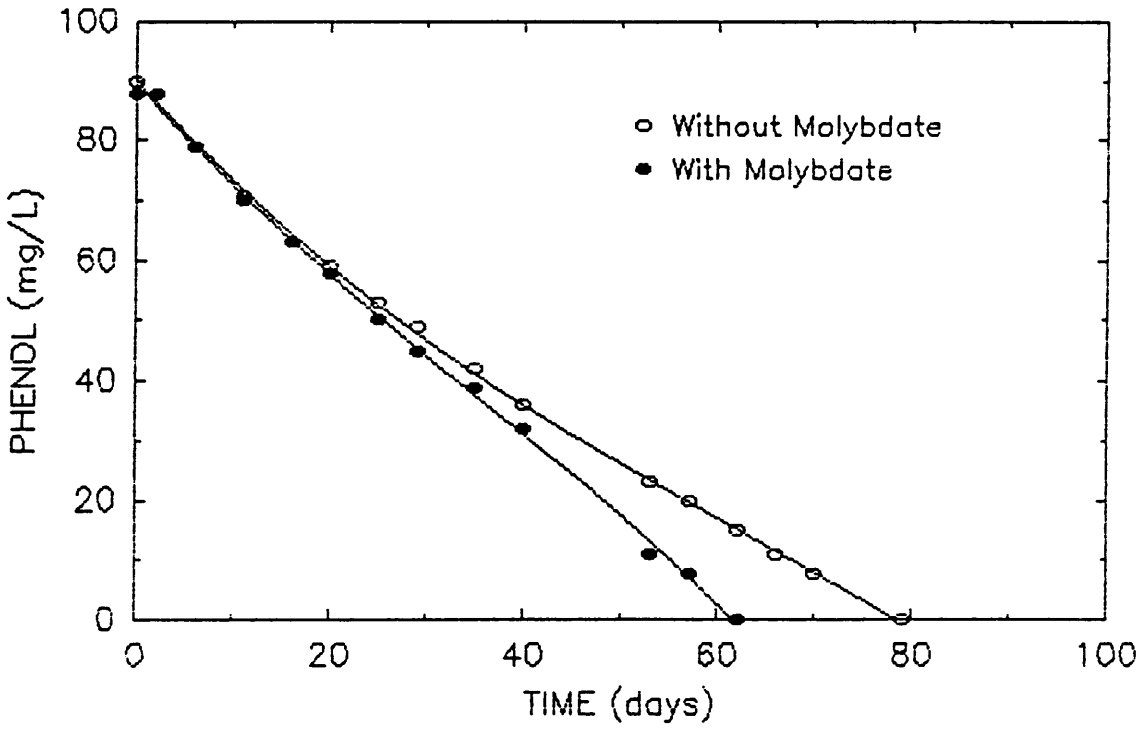

Figure 4.12. Phenol biodegradation in soil microcosms with and without molybdate

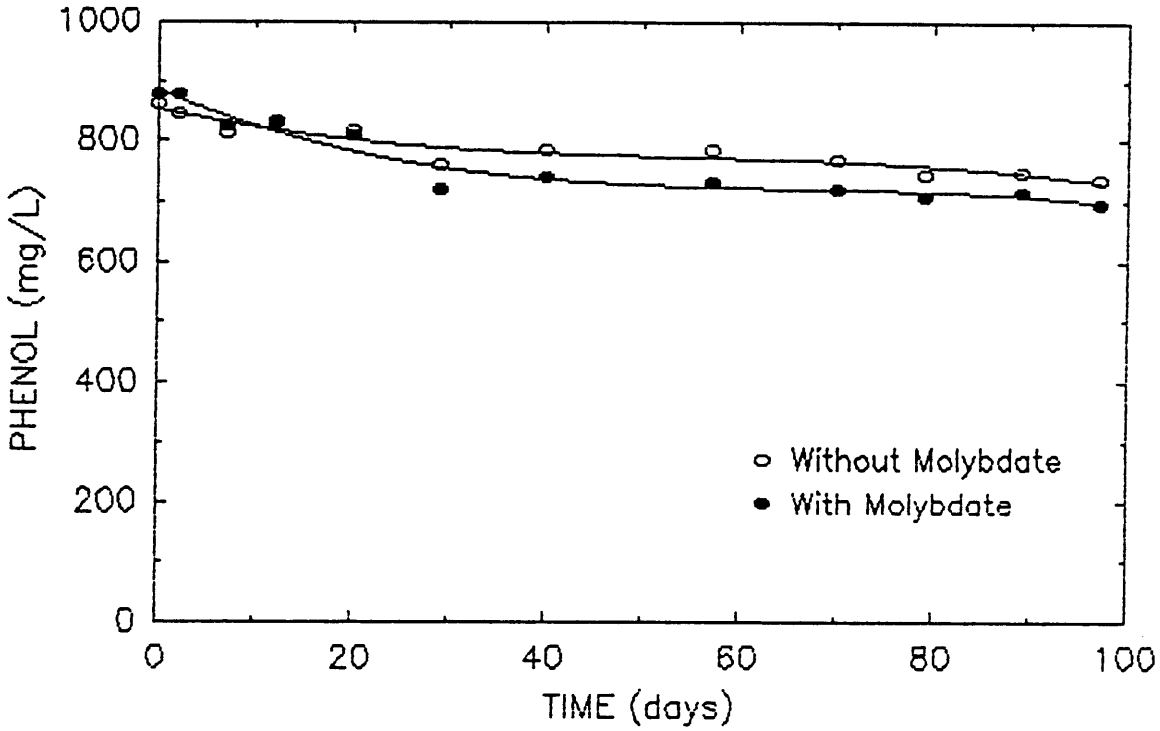

Figure 4.13. Phenol biodegradation in soil microcosms with and without molybdate

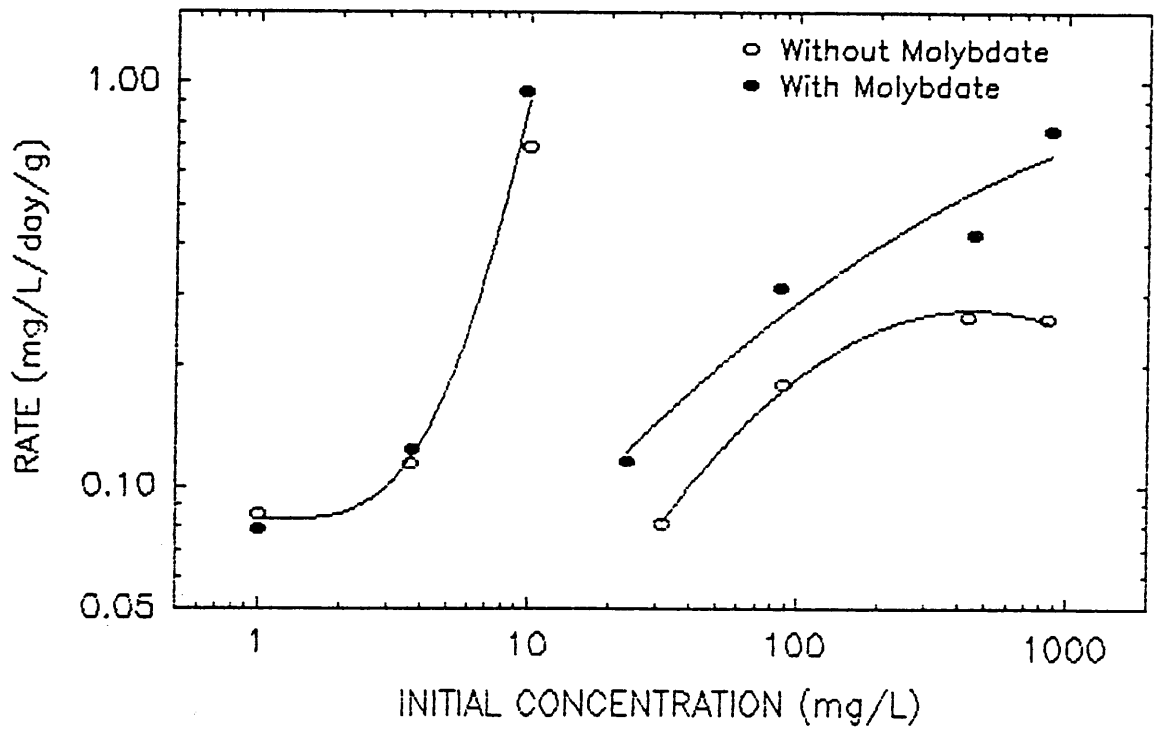

Figure 4.14. Phenol utilization rate in soil microcosms with and without molybdate

Figure 4.14 presents the biodegradation rate data from all of the phenol microcosms. Biodegradation rates from duplicate microcosms were averaged and plotted as single points in this figure. As shown in Figure 4.14, the biodegradation rate increased as the phenol initial concentration increased up to a concentration of 10 mg/L. Above 10 mg/L, the biodegradation rate decreased before increasing again. Therefore, this data appears to represent two different relationships between the biodegradation rate and initial concentration, possibly due to the degradation effects of two different microbial populations at low and high phenol concentrations. Molybdate had no apparent effect on the biodegradation rate at the 1 mg/L and 3.7 mg/L phenol concentrations, but increased the biodegradation rates (37%-191%) at phenol concentrations above 3.7 mg/L.

4.7 2,4-DCP Results

Figure 4.15 shows the biodegradation data for 2,4-DCP at an initial concentration of approximately 25 mg/L. A lag phase of 8 to 10 days occurred before the biodegradation began. Lag phases of various lengths were noted for most of the 2,4-DCP concentrations examined in this experiment. After this lag phase, the 2,4-DCP biodegraded at a relatively constant rate in most of the microcosms. An initial decrease in 2,4-DCP concentration due to adsorption has been previously reported (Smith, 1984), but this did not seem to occur in most of the microcosms in this study. Once the lag phase ended, molybdate increased the 2,4-DCP biodegradation rate from 0.39 to 0.87 mg L^{-1} day^{-1} g^{-1} at the concentration shown in Figure 4.15.

Figure 4.16 presents the biodegradation rate data from all of the 2,4-DCP microcosms. Biodegradation rates from duplicate microcosms were averaged and plotted

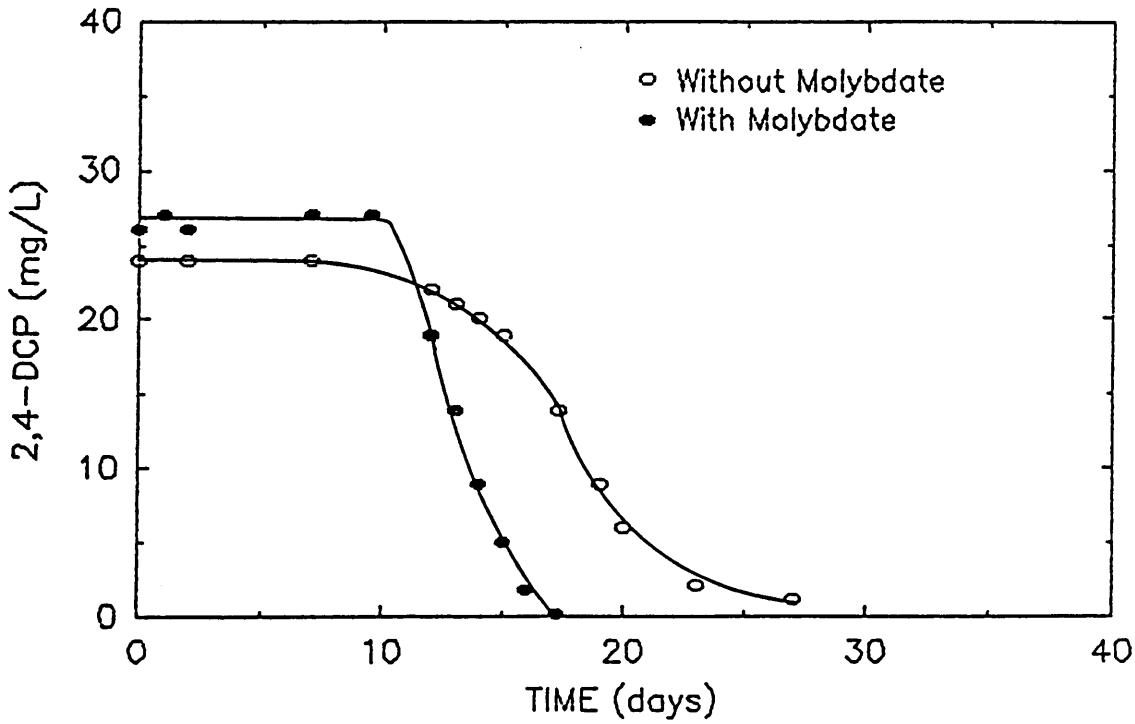

Figure 4.15. 2,4-DCP biodegradation in soil microcosms with and without molybdate

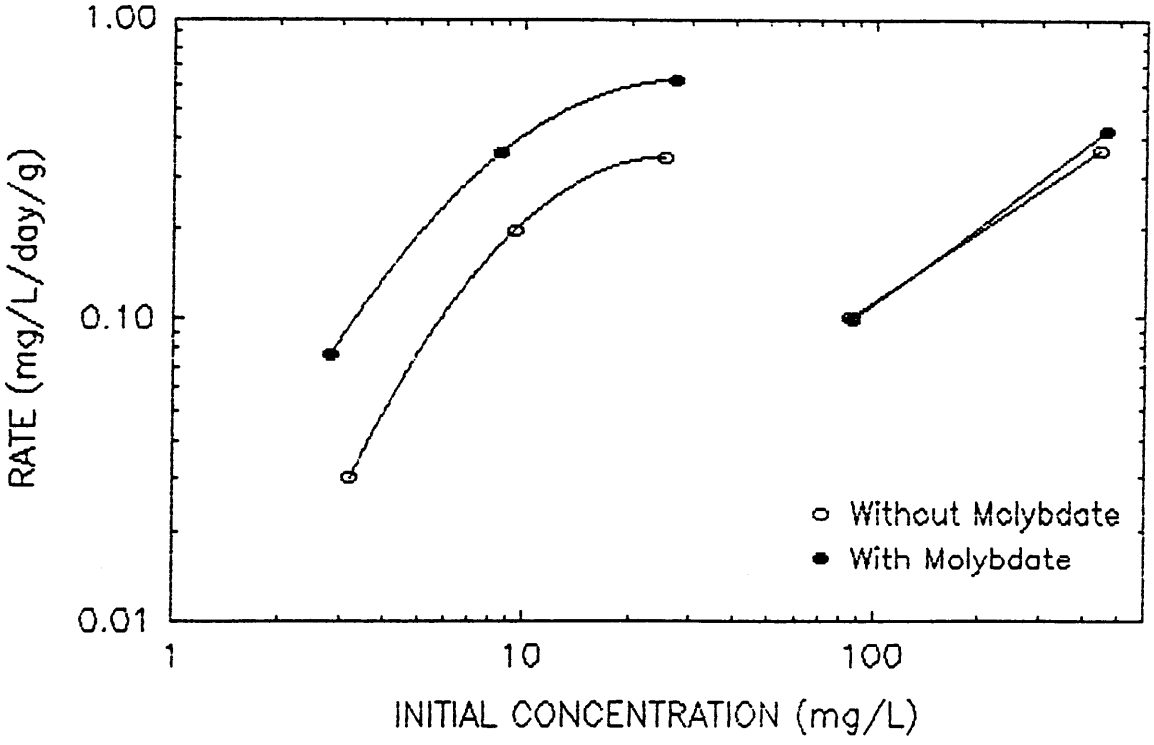

Figure 4.16. 2,4-DCP utilization rate in soil microcosms with and without molybdate

as single points in this figure. The data in Figure 4.16 are shown as representing two different relationships between the degradation rate and the 2,4-DCP initial concentration. The biodegradation rate increased as the 2,4-DCP initial concentration increased up to a concentration of 30 mg/L. Above 30 mg/L, the biodegradation rate decreased (at 85 mg/L) and then increased (at 450 mg/L). Molybdate increased the biodegradation rates (81%-157%) up to a 2,4-DCP concentration of 30 mg/L, but above this concentration molybdate had no effect upon biodegradation rates. Therefore, this data appears to represent two different relationships between the biodegradation rate and initial concentration, possibly due to the degradation effects of two different microbial populations at the lower (3 mg/L - 30 mg/L) and the higher (85 mg/L - 450 mg/L) 2,4-DCP concentration ranges.

Chapter 5

Discussion

5.1 Introduction

Morris (1988) studied the effect of selectively inhibiting either the SRB (with molybdate) or the MB (with BESA) on biodegradation of methanol, TBA, phenol, and 2,4-DCP in subsurface soil samples from Blacksburg, Virginia. Inhibition of the MB decreased the biodegradation rates by up to 70% whereas inhibition of the SRB consistently increased the biodegradation rates (Table 5.1). As shown in Table 5.1, the magnitude of response to molybdate addition was significantly greater for TBA and 2,4-DCP than for phenol and methanol. Morris postulated that anaerobic biodegradation of TBA and 2,4-DCP by MB may require structural substitution of hydrogen as a first step. Therefore, inhibition of the SRB increased the biodegradation rates of TBA and 2,4-DCP more than the rates of the other compounds because the MB can compete for hydrogen to use for structural substitutions as well as reducing equivalents. Morris also determined the biodegradation rate of TBA over a 1-10,000 mg/L concentration range with and without molybdate and found that the largest rate increase with molybdate occurred at the lowest TBA concentration. The rates with and without molybdate converged as the initial TBA concentrations increased.

The primary objective of this study was to investigate the proposed use of hydrogen as a structural substitute or as reducing equivalents in the subsurface anaerobic biodegradation of methanol, MTBE, toluene, phenol, and 2,4-DCP. Another

Table 5.1
Effect of BESA and Molybdate on the Anaerobic Biodegradation Rates of Selected Compounds

Compound	Initial Concentration *(mg/L)*	Effect of BESA *(% rate decrease)*	Effect of Molybdate *(% rate increase)*
Methanol	18	49	32
Phenol	12	30	70
2,4-DCP	12-16	70	307
TBA	20	-	1390

Source: Morris, 1988

objective of this study was to determine if these compounds follow the same biodegradation rate patterns as was observed for TBA. The final objective was to determine the anaerobic biodegradation rate constants for each of the compounds listed above.

5.2 *Methanol Biodegradation and Kinetics*

The following reactions have been suggested for the biodegradation of methanol by methanogens (Lovley and Klug, 1983; Oremland et al., 1982):

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O \quad (5.1)$$

$$CH_3OH + H_2 \rightarrow CH_4 + H_2O \quad (5.2)$$

A comparison of equations 5.1 and 5.2 shows that methanol biodegradation to methane may occur with or without the use of hydrogen. SRB are known to compete with MB for hydrogen (see Section 2.14), but whether SRB compete with MB for methanol is contradictory in the literature (see Section 2.8). Several studies have found that methanol is a noncompetitive substrate (Beeman and Suflita, 1987; Lovley and Klug, 1983b; Oremland and Polcin, 1982; Oremland et al., 1982), and the results from this study support this finding.

The data in Figure 4.2 indicate that the inhibition of the SRB with molybdate slightly increased the methanol biodegradation rate at methanol concentrations below 400 mg/L and had no effect on the biodegradation rate at higher methanol concentrations. Therefore, it appears as if some competition may be occurring between the SRB and the MB for hydrogen at methanol concentrations below 400 mg/L, but that

this competition does not occur at methanol concentrations above 400 mg/L. Molybdate increased the methanol biodegradation rate by 45% at the 8 mg/L methanol concentration. This is in close agreement with the data presented by Morris (1988), which indicated a 32% increase in methanol biodegradation in molybdate amended microcosms containing Blacksburg soil and 18 mg/L methanol.

The reaction order and kinetic rate constants determined for methanol are shown in Table 5.2. A reaction order of approximately 0.15 was determined for methanol biodegradation with and without molybdate by using the log-log plots in Figure 4.2. The methanol biodegradation rate data were linearized by using the Hanes equation over the methanol concentration range of 8-3100 mg/L. This method gave values for the maximum substrate utilization rate (k) and the saturation constant (K_s) for methanol without molybdate amendment of 0.70 mg L^{-1} day^{-1} g^{-1} and 34 mg L^{-1}, respectively. For methanol with molybdate amendment, the values of k and K_s are 0.57 mg L^{-1} day^{-1} g^{-1} and 37 mg L^{-1}, respectively. Therefore, the kinetic constants k and K_s are essentially the same for methanol biodegradation in Blacksburg soil with and without molybdate. Given these values for k and K_s and the long retention times which are common in subsurface soil systems, the complete degradation of methanol in Blacksburg aquifers would be expected.

5.3 MTBE and Organic Amended MTBE Biodegradation and Kinetics

MTBE biodegraded slowly in Blacksburg soil, following a zero order rate in all of the individual microcosms. However, the utilization rate appeared to be first order with respect to initial concentration (Figure 4.6). These rate relationships are the same

Table 5.2
Summary of Reaction Orders and Kinetic Rate Constants for Methanol in Blacksburg Soil

Microcosm Contents	Reaction Order n[a]	Maximum Substrate Utilization Rate k[b] *(mg/L day g)*	Saturation Constant K_s^b *(mg/L)*
Methanol	0.15	0.70	34
Methanol + Molybdate	0.15	0.57	37

[a]Determined for a methanol concentration range of 8-7880 mg/L

[b]Determined for a methanol concentration range of 8-3100 mg/L

as reported by Morris (1988) for TBA. This TBA data is reproduced in Figure 5.1. One obvious difference between the MTBE data shown in Figure 4.3 and the TBA data shown in Figure 5.1 is the effect of molybdate on the utilization rate. Molybdate had almost no effect on the MTBE utilization rate at any of the initial concentrations tested. In contrast, the TBA utilization rate was increased as much as 1390% by the presence of molybdate.

As stated previously, molybdate is a known inhibitor of SRB, and its presence may allow the methanogenic bacteria to successfully compete with the SRB for hydrogen. No biodegradation pathway for TBA has been reported, but Morris (1988) speculates that TBA biodegradation may be analogous to trimethylamine biodegradation (Hippe et al., 1979) and, therefore, may involve structural substitution by hydrogen. The following equation was shown in Chapter 2 as a general biodegradation cleavage reaction for ethers in soil (Alexander, 1981):

$$ROCH_2R' \rightarrow ROH \qquad (2.1)$$

If this general reaction is true for MTBE biodegradation, then a direct structural substitution by hydrogen is also necessary for MTBE biodegradation. Therefore, MTBE and TBA biodegradation should be similarly affected by the presence of molybdate.

The reason for the difference in molybdate effects between MTBE and TBA is not obvious. One speculation is that the hydrogen concentration in the soil which was used in the MTBE microcosms was too low to allow competition between the SRB and the MB to take place. The basis for this speculation involves thermodynamic considerations which are discussed below in relation to ethanol biodegradation (because no specific path for MTBE biodegradation could be found in the literature).

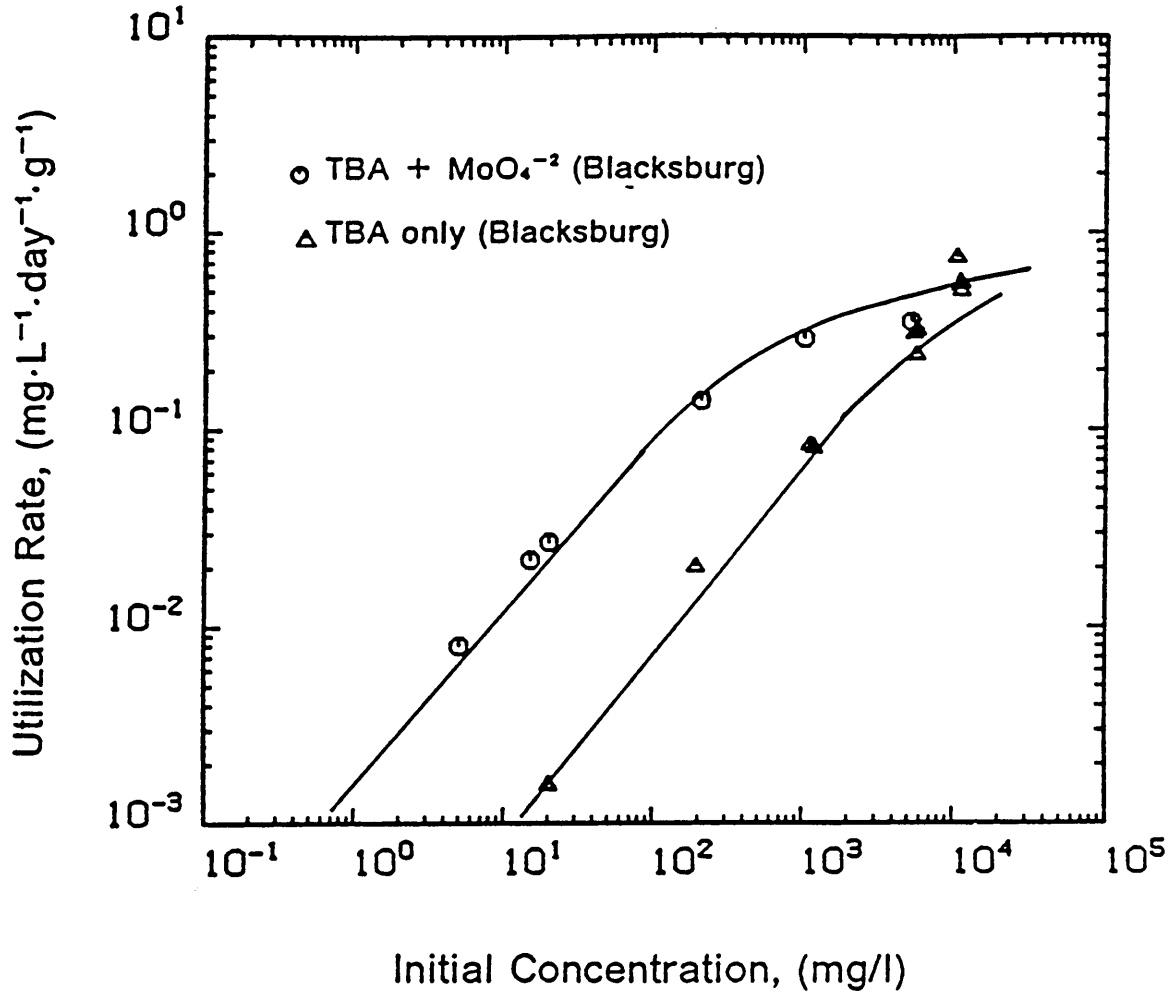

Figure 5.1. Composite of the TBA utilization response in Blacksburg soil with and without molybdate
Source: Morris, 1988

The three reactions which are involved in the conversion of ethanol to methane are shown in Table 5.3 along with the standard Gibbs free energy for each reaction and for the net reaction (McCarty and Smith, 1986). As shown in Table 5.3, the net reaction has a negative standard Gibbs free energy and is therefore thermodynamically favored. Although the initial ethanol reaction has a positive standard Gibbs free energy, the concentration of either acetate or hydrogen could be reduced sufficiently to make this reaction exergonic. McCarty and Smith (1986) have reported that it is the hydrogen concentration which is most important in the control of anaerobic wastewater treatment processes. Above a certain maximum hydrogen concentration, the initial ethanol reaction is not thermodynamically favored. But there is also a minimum hydrogen concentration below which the hydrogen equation in Table 5.3 attains a positive Gibbs free energy and is therefore no longer thermodynamically favored.

The change in Gibbs free energy with hydrogen concentration is illustrated in Figure 5.2 for ethanol biodegradation (McCarty and Smith, 1986). Note that the existence of a minimum and maximum hydrogen concentration for ethanol biodegradation is due to the thermodynamic favorability of the ethanol and the hydrogen reactions (the acetate reaction does not involve hydrogen). Hydrogen experiences a rapid turnover in the system shown in Table 5.3 so the minimum hydrogen concentration is easily reached (McCarty and Smith, 1986).

It is generally agreed that methane formation from complex organics is a three-stage process (Figure 5.3) analogous to the ethanol biodegradation process described above (McCarty and Smith, 1986). Therefore, it is reasonable to speculate that MTBE biodegradation takes place according to a similar process and that a minimum hydrogen concentration is attainable below which no methanogenic

Table 5.3
Methanogenic Conversion of Ethanol

Conversion of ethanol to methane	$\Delta G^{o\prime}$ kJ
Ethanol	
$CH_3CH_2OH(aq) + H_2O(l) = CH_3COO^-(aq) + H^+(aq) + 2H_2(g)$	9.65
Hydrogen	
$2H_2(g) + \frac{1}{2}CO_2(g) = \frac{1}{2}CH_4(g) + H_2O(l)$	-65.37
Acetate	
$CH_3COO^-(aq) + H^+(aq) = CH_4(g) + CO_2(g)$	-35.83
Net	
$CH_3CH_2OH(aq) = \frac{3}{2}CH_4(g) + \frac{1}{2}CO_2(g)$	-91.55

Source: McCarty and Smith, 1986

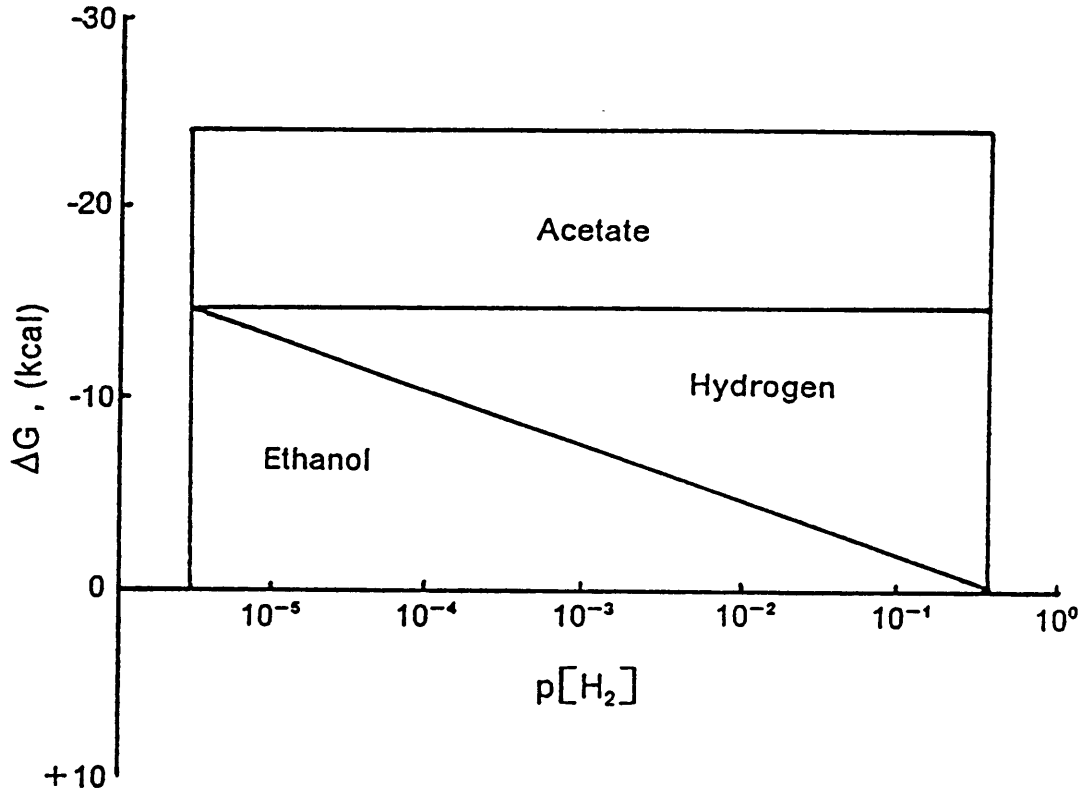

Figure 5.2. Free energy versus H_2 partial pressure for ethanol
Source: McCarty and Smith, 1986

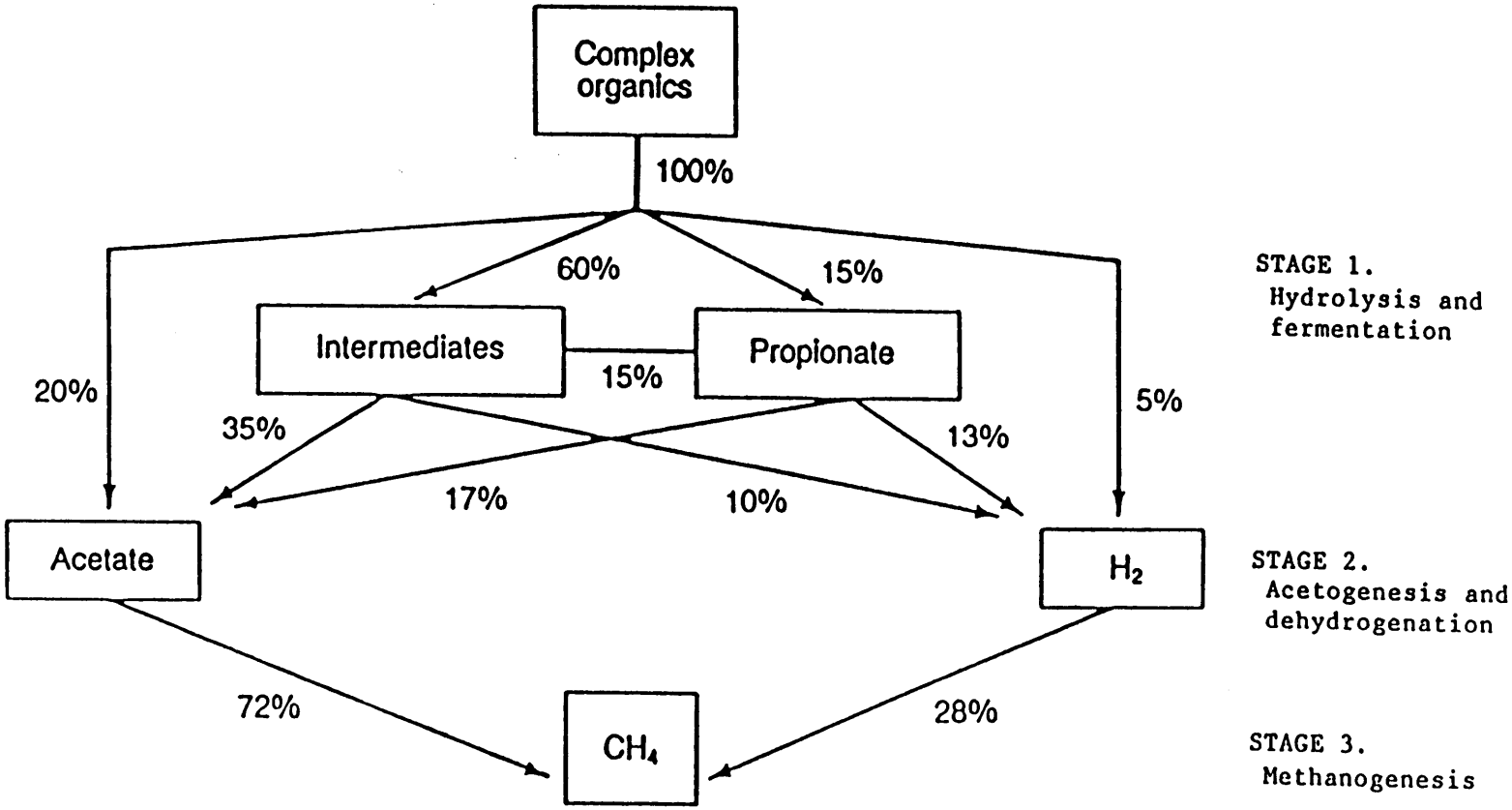

Figure 5.3. Methanogenic decomposition process
Source: McCarty & Smith, 1986

biodegradation will take place. It may be further speculated that SRB will still be active even at this minimum concentration for MB activity. This is illustrated in Figure 5.4 for MTBE and TBA. Note that at hydrogen concentration A, the SRB are still active while there is no degradation of MTBE or TBA by the MB. At hydrogen concentration B, competition occurs between the SRB and the MB for hydrogen. The SRB maintain a higher metabolic rate than the MB at concentration B because the half saturation constants (K_s) for hydrogen are lower for SRB than for MB and, therefore, the SRB have a higher affinity for hydrogen (Hickman, 1988; Kristjansson et al., 1982; Speece, 1983).

To test this theory, the MTBE microcosms were dosed with 30 mg/L of acetic acid and 30 mg/L ethanol in order to provide a source of hydrogen for the MTBE biodegradation reaction. Upon further investigation, it was discovered that the net hydrogen generation upon acetic acid biodegradation is zero (McCarty and Smith, 1986). Therefore, the microcosms were dosed a second time with 30 mg/L ethanol after the first dose of ethanol was completely degraded. Upon addition of ethanol, the biodegradation rates in the molybdate amended microcosms increased, but the biodegradation rates in the microcosms without molybdate remained approximately the same. Since molybdate has been shown to inhibit SRB and thereby allow MB to compete for available hydrogen, it may be assumed that this also occurred in the molybdate amended MTBE microcosms after ethanol addition. The resulting data (Figure 4.8) follows the same pattern as the data reported by Morris for TBA (Figure 5.1). Based upon this data, it seems reasonable to speculate that the MTBE microcosms were originally deficient in hydrogen and that the addition of ethanol provided sufficient hydrogen for MTBE degradation to take place.

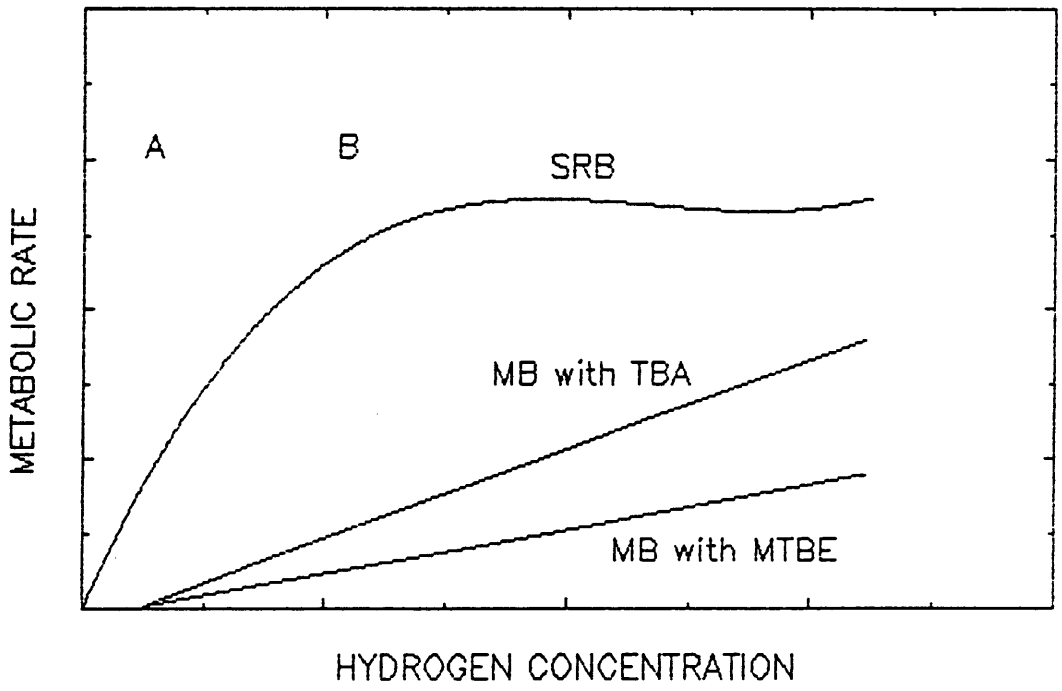

Figure 5.4. Comparison of the metabolic rates of SRB and MB at various hydrogen concentrations

It is possible that many compounds, such as MTBE, are recalcitrant in anaerobic subsurface environments because of a deficiency of hydrogen. Addition of molybdate to such systems would allow the MB to outcompete the SRB only if a certain threshold of hydrogen was available for hydrogen utilization by the MB (Lovley et al., 1982). The data presented above for the organic amended MTBE microcosms indicate that biodegradation of recalcitrant compounds may be stimulated by the addition of organics which produce hydrogen upon biodegradation. More research is needed to fully establish this method for biorestoration of contaminated subsurface environments.

The data for MTBE and organic amended MTBE could not be linearized by the Lineweaver-Burke or the Hanes equations and does not fit the Michaelis-Menton equation. This was evidenced by very low correlation coefficients ($r < 0.7$) upon plotting the data. Therefore, no values for k or K_s are reported for MTBE from the data in this study.

5.4 Toluene Biodegradation and Kinetics

The biodegradation rate pattern for toluene shown in Figure 4.11 is different from the patterns for methanol and MTBE but is similar to the patterns of the other aromatic compounds in this study. The data in Figure 4.11 appear to represent two different rate vs. concentration relationships. Toluene biodegradation rates increased with increasing concentration up to a concentration of 20-40 mg/L, but above the 40 mg/L concentration the rates decreased before increasing again. There is no explanation offered in the literature for this discontinuous relationship. One speculation is that two different microbial populations are responsible for toluene biodegradation over the concentrations studied. It may be that the bacterial population responsible for

biodegradation up to 40 mg/L of toluene becomes inhibited above this concentration. The effects of a second microbial population would then be evident at the higher toluene concentrations. More research is necessary to fully establish this relationship.

Molybdate only increased the biodegradation rate at one of the toluene concentrations (approximately 20 mg/L). It is difficult to explain why molybdate increased the biodegradation rate at one concentration, had no effect on the biodegradation rates at two concentrations (8 mg/L and 40 mg/L) and caused a substantial decrease in biodegradation rates at other concentrations (100-250 mg/L). Scatter in biodegradation data may be due to undetected pockets of air in the microcosm soil (Goldsmith, 1985) or to clumps of bacteria which enter some microcosms but do not enter others (Thornton-Manning et al., 1987). However, data scatter does not explain the consistent failure of molybdate to increase the biodegradation rates. As discussed previously for MTBE, it may have been that the soil in the toluene microcosms was hydrogen limited. A source of hydrogen was not added to the toluene microcosms in this study as was done to the MTBE microcosms. This would be a useful area for future research.

Toluene biodegradation followed a zero order rate in all of the individual microcosms, but the biodegradation reaction order varied from 0.70 to 0.38 (Table 5.4). Since the data in Figure 4.11 is discontinuous, it was necessary to calculate rate constants for two separate concentration ranges. The data was linearized with use of the Hanes equation, and the resulting rate constants are given in Table 5.4. The low value of k and the high value of K_s in the 9-40 mg/L and the 100-260 mg/L toluene concentration ranges indicate that toluene would persist for long periods of time in Blacksburg soil if introduced at these concentration ranges. Molybdate had no effect

Table 5.4
Summary of Reaction Orders and Kinetic Rate Constants for Toluene in Blacksburg Soil

Microcosm Contents	Concentration Limits *(mg/L)*	Reaction Order n	Maximum Substrate Utilization Rate k *(mg/L day g)*	Saturation Constant K_s *(mg/L)*
Toluene	9-40	0.70	0.16	57
Toluene	110-260	0.38	0.07	104
Toluene + Molybdate	9-40	0.70	0.16	57
Toluene + Molybdate	100-220	1.23	—[a]	—[a]

[a]Cannot determine these constants with the Lineweaver-Burke or Hanes linearization methods because the resulting k is negative

on the k or K_s for the lower concentration range (it was not possible to determine rate constants for the molybdate amended microcosms in the 100-220 mg/L concentration range because the resulting k was negative). As mentioned above, it would be very useful to amend microcosms like these with a hydrogen donating organic compound in order to determine the effect of molybdate when sufficient hydrogen is available.

5.5 Phenol Biodegradation and Kinetics

The biodegradation rate pattern for phenol shown in Figure 4.14 is similar to the biodegradation patterns of the other aromatic compounds in this study. The data in Figure 4.14 appear to represent two different rate vs. concentration relationships. Phenol biodegradation rates increased with increasing concentration up to a phenol concentration of 10 mg/L, but above the 10 mg/L concentration the rate decreased before increasing again. As was previously postulated for toluene biodegradation, two different microbial populations may have been responsible for the 2,4-DCP biodegradation over the concentration range studied. If this is so, then the bacterial populations responsible for biodegradation up to 10 mg/L of phenol may have been inhibited above this concentration, and the effects of a second microbial population may then have become evident. In pure cell culture studies, 64 mg/L phenol was found to inhibit cell multiplication of the aerobic bacterium *Pseudomonas putida* (Verschuerenm, 1977). Therefore, inhibition of specific anaerobic bacteria at specific phenol concentrations would not be unexpected. However, much more work would be required to positively establish the proposed causal relationship.

Smith (1984) presented data for phenol biodegradation without molybdate in two unsaturated soil types. A log-log plot of the phenol biodegradation rate data produced

a linear plot with increasing initial concentrations producing increasing reaction rates. No discontinuity was noted in the log-log plot for phenol concentrations ranging from 10 to 1000 mg/L. However, these soils were obtained from sites in Williamsport, Pennsylvania and Dumfries, Virginia, and the microbial communities present in these soils may be different than that of the Blacksburg soil. The reaction order was determined to be approximately first order (n = 0.72-0.78) for the entire 10-1000 mg/L phenol concentration range in the Smith study. Similarly, the data in Figure 4.14 show approximate first order kinetics (n = 0.92) for the 1-10 mg/L phenol concentration range with and without molybdate. In the 20-870 mg/L phenol concentration range, the biodegradation reaction order varied from 0.36 to 0.49 (Table 5.5).

As shown in Figure 4.14, molybdate had no apparent effect on the biodegradation rate at the 1 mg/L and 3.7 mg/L phenol concentrations, but increased the biodegradation rates at phenol concentrations above 3.7 mg/L. Molybdate increased the phenol biodegradation rate by 37% at the 10 mg/L phenol concentration. This is of the same magnitude as the data presented by Morris (1988), which indicated a 70% increase in phenol biodegradation in molybdate amended microcosms containing Blacksburg soil and 12 mg/L phenol.

Kinetic rate constants were determined from the phenol data using the Lineweaver-Burke method (for 1-10 mg/L phenol) and the Hanes method (for 20-870 mg/L phenol). These rate constants are shown in Table 5.5. The low value of K_s for the 1-10 mg/L phenol range indicates that the maximum substrate utilization rate will be achieved at low concentrations so that phenol should not persist in Blacksburg soil if introduced in this concentration range. At the higher phenol concentration range (20-870 mg/L), the K_s is substantially higher, and the maximum substrate utilization rate

Table 5.5
Summary of Reaction Orders and Kinetic Rate Constants for Phenol in Blacksburg Soil

Microcosm Contents	Concentration Limits *(mg/L)*	Reaction Order n	Maximum Substrate Utilization Rate k *(mg/L day g)*	Saturation Constant K_s *(mg/L)*
Phenol	1-10	0.92	0.33	3.0
Phenol	30-850	0.36	0.28	59
Phenol + Molybdate	1-10	0.92	0.44	4.9
Phenol + Molybdate	20-870	0.49	0.85	209

is achieved at much higher concentrations in this range. However, given the values of k and K, in Table 5.5 and the long retention times which are common in subsurface soil systems, the complete degradation of phenol in Blacksburg aquifers would be expected.

5.6 2,4-DCP Biodegradation and Kinetics

The biodegradation rate pattern for 2,4-DCP shown in Figure 4.16 is similar to the patterns of the other aromatic compounds in this study. The 2,4-DCP biodegradation rates increased with increasing concentration up to a concentration of approximately 30 mg/L, but above this concentration the rate decreased before increasing again. As was previously postulated for toluene and phenol biodegradation, two different microbial populations may have been responsible for the 2,4-DCP biodegradation over the concentration range studied. If this is so, then the bacterial population responsible for biodegradation up to 30 mg/L of 2,4-DCP may have been inhibited above this concentration, and the effects of a second microbial population may have then become evident. In pure cell culture studies, 6 mg/L of 2,4-DCP was found to inhibit cell multiplication of the aerobic bacterium *Pseudomonas putida* (Verschuerenm, 1977). Therefore, inhibition of specific anaerobic bacteria at specific 2,4-DCP concentrations would not be unexpected. However, much more work would be required to establish this causal relationship.

Smith (1984) presented data for 2,4-DCP biodegradation without molybdate in unsaturated subsurface soil. A log-log plot of the 2,4-DCP data produced a linear plot showing an increasing reaction rate with increasing initial concentration. No discontinuity was noted in the log-log plot for 2,4-DCP concentrations ranging from 1 to 100 mg/L. However, this soil was obtained from Dumfries, Virginia, and the

microbial communities present in this soil may have been different than that of the Blacksburg soil. The biodegradation reaction order was determined to be approximately first order (n = 0.90) for the 1-100 mg/L 2,4-DCP concentration range in the Smith study. Similarly, the data in Figure 4.16 show approximate first order kinetics for both of the concentration ranges (Table 5.6).

As shown in Figure 4.16, molybdate increased the 2,4-DCP biodegradation rates across the lower concentration range (3-30 mg/L), but had no effect in the upper concentration range (85-460 mg/L). Molybdate increased the biodegradation rate by 150% at 3 mg/L 2,4-DCP and by 81% at both 9 mg/L and 25 mg/L 2,4-DCP. These are large increases but are not quite as large as the 307% increase reported by Morris (1988) for 12-16 mg/L 2,4-DCP. The lack of effect by molybdate at the upper 2,4-DCP concentrations may indicate that 2,4-DCP biodegradation follows the same pattern at high concentrations as was shown for methanol and MTBE in this study and for TBA in the Morris study, or it may indicate that a different microbial population was responsible for 2,4-DCP biodegradation at the upper concentrations (as was proposed above).

The data for 2,4-DCP could not be linearized by the Lineweaver-Burke or the Hanes equations and does not fit the Michaelis-Menton kinetic equation. Very low correlation coefficients ($r < 0.6$) and negative k values were obtained upon plotting of the data. Therefore, no values for k or K_s are reported for 2,4-DCP from the data in this study.

Table 5.6
Summary of Reaction Orders Determined for 2,4-DCP in Blacksburg Soil

Microcosm Contents	Concentration Limits *(mg/L)*	Reaction Order n
2,4-DCP	3-25	1.75
2,4-DCP	85-440	0.78
2,4-DCP + Molybdate	3-27	1.38
2,4-DCP + Molybdate	87-460	0.87

5.7 The Effect of Molybdate Addition Upon Biodegradation Rates

The primary objective of this study was to investigate the proposed use of hydrogen as a structural substitute or as reducing equivalents in the subsurface anaerobic biodegradation of methanol, MTBE, toluene, phenol, and 2,4-DCP. It has been proposed that molybdate inhibits the SRB and thereby allows the MB to effectively compete for hydrogen. Compounds requiring hydrogen substitution as part of the biodegradation mechanism should experience greater increases with molybdate addition than would compounds which require hydrogen only as a reducing equivalent. Therefore, one way to accomplish the primary objective of this study was to determine the increase in biodegradation rates caused by the addition of molybdate to microcosms containing the compounds of interest and to compare these increases between compounds.

Since the concentrations of man-made organics rarely exceeds 10 mg/L in contaminated subsurface soils, the biodegradation rates will be compared at the 10 mg/L concentration. The percent increases in biodegradation rates due to the presence of molybdate are as follows: organic amended MTBE, 229%; 2,4-DCP, 81%; MTBE, 72%; methanol, 45%; phenol, 37%; toluene, 0%. The actual rate increases are shown in Figure 5.5. As discussed earlier, the MTBE microcosms were amended with ethanol because the initial MTBE results indicated that these microcosms were hydrogen deficient. As the ethanol in these microcosms biodegraded, the biodegradation rate in the molybdate amended microcosms increased while the rate in the microcosms without molybdate remained approximately the same. If MTBE degrades by the mechanism shown earlier in Equation 2.1, then hydrogen substitution is part of MTBE

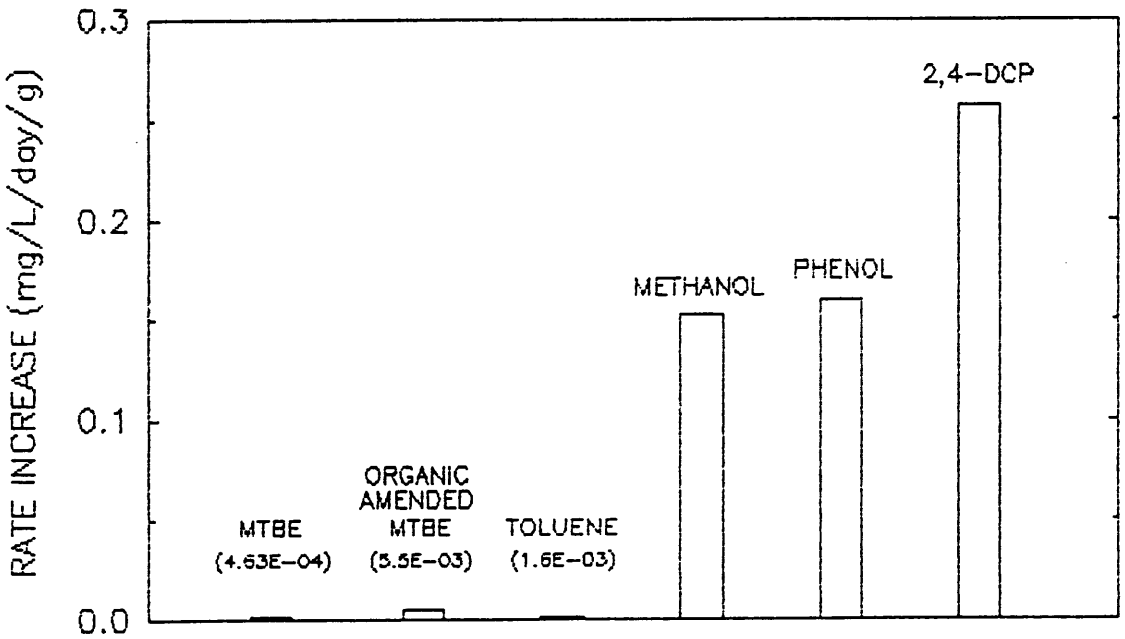

Figure 5.5. Increase in biodegradation rate with molybdate addition (1.0 mM MoO_4^{2-}) for compounds at 10 mg/L concentration: Numbers in parentheses are given to permit comparison of low rate increases.

biodegradation, and the 229% increase in the MTBE biodegradation rate upon molybdate addition would be expected. Biological dehalogenation of 2,4-DCP by hydrogen substitution has been reported (Gibson and Suflita, 1986; Suflita and Miller, 1985), so the 81% increase in the 2,4-DCP biodegradation rate would also be expected. Morris (1988) noted a 307% increase in 2,4-DCP biodegradation with molybdate addition at 14-16 mg/L 2,4-DCP concentrations. This is somewhat larger but of the same order of magnitude as the 157% maximum increase noted in the present study for the 3 mg/L 2,4-DCP concentration.

Young and Rivera (1985) used high performance liquid chromatography to determine the metabolites in the anaerobic biodegradation of phenol. They found that ring saturation takes place, followed by ring fission, yielding organic acid intermediates which serve as methane precursers. Hydrogen is required to saturate the phenolic ring, so an increase in biodegradation rate would be expected if the MB are given an advantage in the competition for hydrogen. Phenol biodegradation behaved differently than biodegradation of the other compounds in that the maximum increase in the biodegradation rate with molybdate addition (191%) occurred at the highest concentration tested. The average increase in phenol biodegradation rate upon addition of molybdate was 80%, and this average increase compares favorably with the 70% increase reported by Morris (1988) for 12 mg/L phenol.

The percent increase shown above for toluene may be misleading in that molybdate only increased the biodegradation rate of toluene for one set of microcosms (two microcosms per set) at 20-25 mg/L. At the other toluene concentrations, molybdate either had no effect or decreased the biodegradation rate. This data is puzzling since the biodegradation of toluene involves ring saturation (Grbic-Galic and

Vogel, 1987) and thus requires hydrogen. Therefore, the effect of molybdate on toluene biodegradation should be similar to that of phenol. As previously mentioned for MTBE, the soil in the toluene microcosms may have been hydrogen deficient, and therefore no increase in degradation rate with molybdate addition would occur. A source of hydrogen was not added to these microcosms as was done to the MTBE microcosms. This would be a useful area for future research. The effect of molybdate on methanol biodegradation was as expected since methanol can degrade directly to methane either with or without hydrogen substitution (Equations 5.1 and 5.2), and since several studies have indicated that methanol is a noncompetitive substrate (Beeman and Suflita, 1987; Lovley and Klug, 1983b; Oremland and Polcin, 1982; Oremland et al., 1982).

The biodegradation rate data presented in this study for methanol, MTBE, phenol, and 2,4-DCP seem to confirm the hypothesis proposed by Morris (1988) that inhibition of sulfate reduction by molybdate may enhance biodegradation more for compounds which require hydrogen for structural substitution than for those compounds which require hydrogen as a reducing equivalent for biodegradation. However, molybdate addition would increase the biodegradation rate only if a certain threshold of hydrogen was available for hydrogen utilization by the MB (Lovley et al., 1982). The data presented for organic amended MTBE microcosms indicate that biodegradation of recalcitrant compounds may be stimulated in hydrogen deficient environments by the addition of organics which produce hydrogen upon biodegradation. More research is needed to fully establish this method for biorestoration of contaminated subsurface environments.

Chapter 6

Conclusions

The primary objective of this study was to investigate the role of hydrogen as a structural substitute or as a reducing equivalent in the anaerobic biodegradation of methanol, MTBE, toluene, phenol, and 2,4-dichlorophenol. In addition, the biodegradation rates of these compounds at various initial concentrations with and without inhibition of sulfate reducing bacteria were determined along with anaerobic biodegradation rate constants for each of the compounds studied.

From the results obtained in this study, the following conclusions can be made:

1. Rates of methanol biodegradation were slightly altered in molybdate amended microcosms indicating that methanol is a noncompetitive substrate in Blacksburg soil.
2. MTBE biodegradation in Blacksburg soil was slow, and molybdate addition had no effect on MTBE biodegradation. This was attributed to the hydrogen level being below the lower hydrogen threshold for methanogenic bacterial activity.
3. Although the rates were still low, molybdate increased the biodegradation rate in MTBE microcosms which were amended with ethanol. This was attributed to an increase in methanogenic bacterial activity caused by an increase in hydrogen concentration upon ethanol biodegradation.
4. Toluene, phenol, and 2,4-dichlorophenol biodegradation proceeded at two different rate versus concentration relationships for lower and upper concentration ranges, possibly due to the presence of two different populations of microorganisms at the two concentration ranges.
5. The addition of molybdate to inhibit sulfate reduction increased the degradation rates more for compounds which may require hydrogen in a structural position (2,4-DCP, MTBE) than those compounds which require hydrogen for proton reduction (methanol).
6. The results of this experiment support the theory that addition of molybdate allows the methanogenic bacteria to outcompete the sulfate reducing

bacteria for hydrogen only when a certain threshold of hydrogen is available for utilization by the methanogenic bacteria. Therefore, anaerobic biodegradation of recalcitrant compounds may be stimulated by the addition of organics (such as ethanol) which produce hydrogen upon biodegradation.

Chapter 7

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